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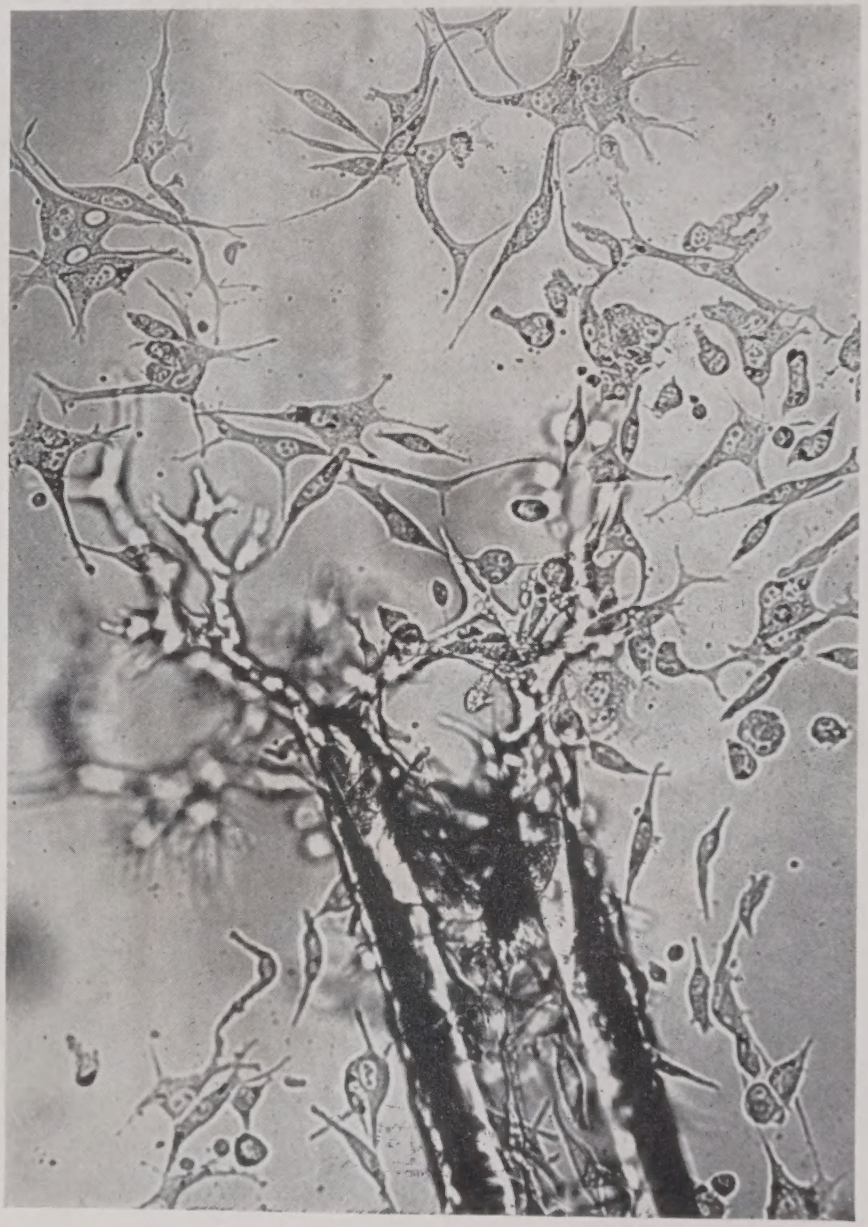

FIG. 1. Earle's pure strain of mouse cells emerging from open end of capillary tube into plasma of outer culture flask twenty-eight days after first cell had been isolated in this same capillary. (K. K. Sanford, W. R. Earle and G. D. Likely.[233]) ×145.

Methods of
TISSUE CULTURE

Raymond C. Parker, Ph.D.
Research Associate, Connaught Medical Research Laboratories, and Associate Professor of Experimental Cytology, School of Hygiene, University of Toronto

With a Chapter by
Joseph F. Morgan, Ph.D.
Research Associate, Connaught Medical Research Laboratories, University of Toronto

SECOND EDITION, REVISED
WITH 113 ILLUSTRATIONS

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TO MY TEACHERS

Ross G. Harrison
Albert Fischer
Alexis Carrel

PREFACE TO THE SECOND EDITION

THE first edition of this book dealt almost exclusively with the techniques and practices that were in use in the laboratory of the late Dr. Alexis Carrel at The Rockefeller Institute for Medical Research, in New York. In the present edition, an attempt has been made to describe the major tissue culture procedures now in general use in many laboratories, and to indicate some of their applications.

While certain sections of the earlier edition have been omitted, the remainder have been reorganized, revised and extended, and many new sections have been added. Thus, the introductory chapter has been revised and expanded to include not only a general survey of the techniques and their applications but also the major results achieved by applying the techniques to various special problems. Chapter IV has been enlarged to include a detailed discussion of the fundamental principles and modern practices of sterilization, and new sections have been written on filtration and on various other means of achieving and safeguarding sterility. Chapter V has been revised to include the historical development of balanced salt solutions, and descriptions are given of some of the newer diluting fluids. Chapters VI and VII include all of the more recent practices and procedures having to do with the preparation of plasma, serum, and tissue extracts. Chapter VIII has been completely rewritten to include the latest information in the field of animal cell nutrition, in which important advances have been made over the last few years.

The next five chapters, which deal with coverslip cultures of various types (Chap. IX), flash techniques for plasma cultures (Chap. X) and for fluid cultures (Chap. XI), roller-tube cultures

(Chap. XII), and flask techniques for tissue fragments in suspension (Chap. XIII), represent a complete reorganization and revision of these topics and include several new procedures that have recently been developed. Chapter XIV is a new section having to do with watch-glass cultures for embryological studies. Chapter XV, which deals with growth measurements and their interpretation, has been extended to include a number of new methods of estimating growth. It is hoped that the completed effort will assist the increasing number of investigators who wish to apply the methods of tissue culture to their own particular problems.

The tissue culture literature has grown tremendously during the last decade. Fortunately, the Tissue Culture Association is preparing a comprehensive bibliography of investigations involving the use of the techniques. It has therefore seemed wise to preserve the compactness and convenience of this volume by omitting the classified list of references, which contained 753 titles in the first edition. The Association's bibliography, which will soon be available, is being prepared under the direction of Dr. Margaret R. Murray, of the College of Physicians and Surgeons of Columbia University, and will comprise some fifteen thousand entries indexed according to author and subject matter.

I wish to record my gratitude to all who have assisted in the revision. I refer particularly to Dr. Joseph F. Morgan, who contributed the new chapter on the development of synthetic media (Chap. VIII) and assisted in gathering new material for Chapters VII and XV; to Dr. Wilton R. Earle, Dr. Albert H. Ebeling, Dr. George O. Gey, Dr. Margaret R. Murray, and Dr. Keith R. Porter, who contributed valuable information and certain of the new illustrations; to Mr. R. P. Loveland, of the Research Laboratories of Eastman Kodak Company, who again read the chapter on Photomicrography and Microcinematography and made useful suggestions for its revision. I am indebted also to Miss J. M. Day and Miss M. M. Imrie, who assisted in preparing the manuscript; to Mr. C. G. Smith and Mrs. Louise Gordon, who assisted with the new illustrations; and, to many others among my colleagues and associates in Toronto who suffered far more than their rightful share while the work was in progress. Finally, I would again express my appreciation of the unfailing courtesy and helpfulness of the publishers.

R. C. P.

Toronto, Canada

PREFACE TO THE FIRST EDITION

OVER the past ten years, much has been written about the methods of tissue culture. The various accounts that have appeared fall into two groups: those that have laid considerable emphasis upon the difficulties involved, without elucidating them sufficiently; and those that have minimized the effectiveness of the various procedures by treating them superficially. Furthermore, the recent accounts are quite incomplete, and the older ones are long out of date. As a result, many students and investigators have become discouraged even before attempting to utilize the methods, whereas others have been led into pitfalls that might otherwise have been avoided.

On the present occasion, an attempt has been made to describe the essential details of the more basic procedures used in Carrel's laboratory at The Rockefeller Institute. And while the presentation may seem somewhat incomplete because it deals almost exclusively with the methods followed in that laboratory, this course has been taken in the firm belief that the procedures described permit more precise standardization and a greater latitude of usefulness than any others now in existence. It is hoped that they have been presented in such manner that they may be applied directly in their present form, or else that they may serve as a basis for further elaborations to meet the special demands of individual requirements.

Since the book is intended both for those who are unacquainted with the more recent developments in tissue culture, and for others who have never had occasion to use the methods in any form, an effort has also been made to give the reader a certain degree of orientation in the extensive literature that has grown up around the use of the various procedures. In Chapter XVI may be found examples of the manner in which the methods have been applied to advantage in five major fields of investigation, namely, experimental morphology, and the study of tumors, viruses, hypersensitivity and immunity. In the classified bibliography at the end of the book, there are supplementary references to experiments in these and other fields of investigation not discussed in the text.

To my colleagues and associates, and all those who have given of their time and assistance, I am profoundly indebted and offer my grateful thanks. I refer, especially, to Dr. Alexis Carrel, for whose interest, advice and criticism I am particularly indebted; to Dr. Albert H. Ebeling, Dr. Lillian E. Baker, Miss Irene E. McFaul, Miss Augusta J. Hollender, Miss Olive M. Johnston, Miss Cesira Macri, Mrs. Walter M. Gladding, Jr., Mr. William La Rosa, and Mr. William Haratonik, all of whom have helped in numerous ways and have saved me from many an embarrassing error in technical detail; to Mr. Charles B. Spies, and his associates, of the Purchasing Division of The Rockefeller Institute, who helped greatly in describing the special equipment mentioned in Chapter III; to Mr. Joseph B. Haulenbeek, and his associates, of the Illustration Division of The Rockefeller Institute, who photographed all displays of equipment and technical procedures, and executed the diagrams; to Dr. Geoffrey Rake, who read the entire manuscript and offered many valuable suggestions; and to Miss Josephine Bates, who prepared the manuscript for publication.

I am also indebted to Dr. William Bloom for the privilege of reading, in manuscript form, his article on "Cellular differentiation and tissue culture"; to Col. Charles A. Lindbergh, for the privilege of describing, for the first time, an apparatus devised by him for the preparation of serum from coagulated plasma, and also a special flask for the continuous circulation of fluid media; to Dr. Warren H. Lewis, for permission to describe his ring-slide technique; and to Mr. R. P. Loveland and Mr. G. E. Matthews of the Research Laboratories of the Eastman Kodak Company, and Mr. E. Keller of E. Leitz, Inc., all of whom read Chapter XV.

Finally, I wish to acknowledge a debt of extreme gratitude to the late Mr. Paul B. Hoeber, of Harper and Brothers, whose interest and encouragement did much to lighten the task; and to his associate, Miss Florence Fuller, who has seen the book through the trials of actual publication.

R.C.P.

New York, N. Y.

CONTENTS

PREFACE TO THE SECOND EDITION vii

PREFACE TO THE FIRST EDITION x

LIST OF ILLUSTRATIONS xvii

I. INTRODUCTION 1

II. THE LABORATORY 39

III. SPECIAL EQUIPMENT 43

IV. CLEANSING AND STERILIZATION PROCEDURES 54

Cleansing procedures 55

Sterilization of materials 58

Filtration 67

Use of face masks 69

Hand sterilization 70

Preparation of Dakin's hypochlorite solution 71

V. DILUTING FLUIDS, *p*H DETERMINATIONS AND OSMOTIC PRESSURE 75

Tyrode's solution 77

Double Tyrode's solution 79

Tyrode's solution with extra glucose 79

Glucosol solution 79

Simms' solution 80

Earle's solution 80

Gey's solution 81

Filtration of diluting fluids 81

Preparation of *p*H standards 83

Determination of *p*H by the drop method 83

Standard flasks for use in determining the *p*H of cultures 85

Cryoscopic (freezing-point) method of determining osmotic pressure 86

VI. PREPARATION OF PLASMA AND SERUM 88

Preparation of paraffined tubes for the storage of blood plasma 88

Preparation of adult chicken plasma 90

Bleeding from the carotid artery 91

Bleeding from the wing 97

Preparation of chicken plasma with an anticoagulant 98

Preparation of mammalian plasma 99

Preparation of serum 102

VII. PREPARATION OF TISSUE EXTRACTS 107

Extracts prepared in a motor-driven Pyrex homogenizer 111

Extracts prepared from hand-chopped tissues 113

VIII. DEVELOPMENT OF SYNTHETIC MEDIA (*by Joseph F. Morgan*) 115

IX. COVERSLIP CULTURES 132

Preparation of hanging-drop cultures 133

Transplantation of hanging-drop cultures 138

Ring-slide cultures 143

Perforated-slide cultures 144

Double-coverslip cultures 147

X. FLASK TECHNIQUES FOR PLASMA CULTURES 148

Preparation of plasma cultures 150

Adjustment of *p*H with carbon dioxide gas 153

Subsequent treatment of plasma cultures 156

Transplantation of plasma cultures 159

Propagation of pure strains by single-cell isolation 160

Cultivation of special cell types 163

Blood leucocytes in plasma medium 166

Cultivation of tissues in heterologous plasma 169

Cultivation of tissues in fibrinogen 170

Use of antitryptic agents to prevent digestion of coagulum 173

Cultivation of fresh tissue explants for assay purposes 175
XI. FLASK TECHNIQUES FOR FLUID CULTURES 179
Blood leucocytes in fluid media 180
Perforated cellophane cultures 188
Flask techniques for continuous circulation of fluid media 190
XII. ROLLER-TUBE TECHNIQUES 196
Special rotating devices 196
Roller-tube cultures without plasma 200
Roller-tube cultures with plasma 202
XIII. FLASK TECHNIQUES FOR TISSUE FRAGMENTS IN SUSPENSION 204
Cultivation of tissues in suspension for physiological studies 205
Cultivation of tissues in suspension for virus propagation 210
XIV. WATCH-GLASS CULTURES FOR EMBRYOLOGICAL STUDIES 215
XV. GROWTH MEASUREMENTS AND THEIR INTERPRETATION 218
Measurement of surface areas 218
Estimation of mitotic coefficients 223
Measurement of dry weights 224
Measurement of total metabolism by manometric methods 226
Measurement of glucose utilization 228
Measurement of lactic acid production 230
Measurement of nitrogen metabolism 231
Measurement of nucleic acid phosphorus 232
Measurement of chemical changes by means of radio-active isotopes 235
XVI. HISTOLOGICAL PROCEDURES 236
XVII. PHOTOMICROGRAPHY AND MICROCINEMATOGRAPHY 259
REFERENCES 271
INDEX 289

LIST OF ILLUSTRATIONS

1. Earle's pure strain of mouse cells emerging from open end of capillary tube into plasma of outer culture flask twenty-eight days after first cell had been isolated in this same capillary *Frontispiece*
2. Marginal area of culture of Carrel's strain of chick embryo spindle cells (fibroblasts) after strain had been propagated for twenty-six years 4
3. Spinal ganglion cells from an eleven-day chick embryo; fixed and stained after five days' cultivation in equal parts of chicken plasma and embryo tissue extract 5
4. Fragment of embryo rat kidney after nine days' cultivation in chicken plasma and rat serum (*A*). Culture of skeletal muscle from an embryo rat showing the development of several well-defined muscle buds (*B*) 6
5. Fringe of new cells surrounding fragments of tissue excised from a culture of chick embryo breast muscle that had been embedded in a plasma coagulum and treated with adult chicken serum for one year before being transferred to a fresh flask (*A*). Stained section through center of a tissue fragment that had been treated with adult chicken serum for one year in a single flask (*B*) 8
6. Tubules from the marginal area of a culture of mouse carcinoma (E-0771) after six months' cultivation in a single flask (*A*). Stained section through the central cluster of tubules shown in *A* (*B*) 9

7. Stained section of fragments of adult chicken spleen after twenty days' cultivation in a fluid medium consisting of 50 per cent rabbit serum in modified Tyrode's solution 11

8. Development of chick embryo femora cultivated on the surface of a clot consisting of equal parts of plasma and embryo tissue extract 12

9. Marginal area of a stained culture of Fischer's strain of Ehrlich mouse carcinoma in the two hundred sixty-second passage (*A*). Four-day-old culture of spontaneous adenoma from the mouse (*B*) 15

10. Malignant cells from dibenzanthracene mouse tumor C 37 removed from an animal after three generations and cultivated for one day in a hanging drop (*A*). Cells from a culture of the tumor shown in *A*, but removed from an animal after twenty-five generations (*B*). Malignant cells from mouse tumor PHS 1 (produced by dibenzanthracene) removed from an animal after twenty-two generations and cultivated for one day in a hanging drop (*C*). Cells from a culture of the tumor shown in *C*, but removed from an animal after thirty-seven generations (*D*) 17

11. Electron micrograph of the margin of a cell from a culture of rat fibroblasts 20

12. Seventeen-day-old double-coverslip culture of human sympathicoblastoma (or neuroblastoma) growing as sheet-like epithelium with neurites and fibroblasts 21

13. Marginal area of culture of cells derived from mouse (Swiss) subcutaneous connective and adipose tissue and cultivated for ten months in 40 per cent Earle's salt mixture, 40 per cent horse serum and 20 per cent chick embryo extract (*A*). Marginal area of culture from another sub-line of the same origin as the cells shown in *A*, after ten months (*B*) 24

14. Cell containing elementary bodies of psittacosis virus 30

15. Four-day culture of connective tissue cells derived from the skeletal muscle of a twelve-day-old chick embryo and cultivated for sixteen passages (weeks) in rabbit plasma 36

16. Stained section of portion of a six-day culture of connective tissue cells derived from the skeletal muscle of a twelve-day-old chick embryo and cultivated for thirty passages (weeks) in rabbit plasma 37

17. Wooden block for the accommodation of tubes being filled with sterile media 44

18. Wooden block for storing and transporting culture media in tubes; also, 50-ml. Erlenmeyer flasks with their stoppers protected from dust by glass and aluminum caps 45

19. Carrel D-3.5 culture flask 46

20. Carrel "micro" culture flask 46

21. Instruments used in tissue culture 47

22. Culture pipettes 48

23. Metal rack for the accommodation of D-3.5 culture flasks 49

24. Metal rack for the accommodation of sterile pipettes and instruments 50

25. Platinum spatulas 52

26. Seitz method of filtration with especially prepared paper discs 68

27. Seitz filtration by suction, and the procedure followed in filling storage tubes 82

28. Procedure followed in paraffining tubes for the storage of blood plasma (*A*). Procedure followed in corking and wrapping the paraffined tubes (*B*) 89

29. Glass bleeding cannulae 92

30. Glass bell-cannula used in bleeding a chicken from the carotid artery 96

31. Large bulb pipette used in transferring plasma from bleeding tubes to paraffined storage tubes 97

32. Device used for bleeding from the carotid artery of a chicken directly into a 250-ml. centrifuge bottle, when an anticoagulant is present 99

33. Double centrifuge tube for the preparation of chicken serum from coagulated plasma 103

34. Diagram showing a method of constructing a Lindbergh double centrifuge tube from sections of brass pipe 104

35. Pyrex homogenizer for use in preparing embryo tissue extracts 112

36. Convenient arrangement of tables, stools and equipment for all types of tissue culture work 134

37. Preparation of hanging-drop cultures 136

38. Hanging-drop cultures being prepared for transfer to new medium 139

39. Incisions made in preparing culture transplants 140

40. Diagram representing the procedure followed in the preparation of ring-slide cultures 143

41. Wooden rack for the accommodation of perforated-slide and double-coverslip cultures; also, a small Coplin jar with vertical grooves to hold four coverslips during washing or staining 146

42. Carrel D-5 culture flask 149

43. Preparation of cultures in Carrel D-3.5 flasks 151

44. Carrel culture flasks: Closed temporarily with rubber caps in order that excess carbon dioxide may be allowed to escape by diffusion (*A*); closed with rubber stoppers (*B*) 155

45. Removal of culture fluid from a D-3.5 flask by suction (*A*). Plasma culture being freed from the bottom of a D-3.5 flask with the aid of a platinum spatula (*B*).

Plasma coagulum after it has been removed from the flask and deposited on a glass plate for cutting (*C*) 157

46. Origin of Earle's pure strain of mouse cells by single-cell isolation 161

47. Eleven-day sister cultures of twelfth passage chick fibroblasts cultivated in a plasma medium containing soybean antitrypsin (*A*); and, in the absence of antitrypsin (*B*) 174

48. Differences in the appearance of leucocytes derived from the blood of four normal chickens and cultivated for seven days in 50 per cent autologous serum and Tyrode's solution 181

49. Human leucocytes derived from the blood of four individuals and cultivated for seven days in 50 per cent autologous serum and Tyrode's solution 182

50. Leucocytes derived from a normal chicken and from one infected with Rous sarcoma, each cell type cultivated for seven days in 50 per cent serum obtained from both the normal and the pathological chicken 184

51. Chicken leucocytes cultivated for ten days in 50 per cent rabbit serum, and in another sample of the same serum heated for one hour at 55° C. (*A* and *B*). Chicken leucocytes cultivated for seven days in 50 per cent chicken serum and Tyrode's solution, and for the same length of time in the same medium to which was added 2.5 mg. per cent thyroxin (*C* and *D*) 185

52. Chick embryo cells cultivated for sixty-five days on perforated cellophane in a fluid medium that contained 40 per cent chicken serum for the first fifteen days and 40 per cent horse serum thereafter 188

53. Second passage chick embryo cells cultivated for fifteen days on perforated cellophane in a fluid medium that contained 40 per cent chicken serum, then for twenty-three days in a medium containing 40 per cent horse serum (*A*). Third passage chick embryo

cells cultivated for eight days in a fluid medium containing chicken serum, then for twenty-one days in a medium containing horse serum (*B*) 189

54. Lindbergh culture flask for the continuous circulation of a large quantity of fluid medium 192

55. Four-year-old strain of human sarcoma cells cultivated in roller tubes 197

56. A small roller-tube incubator with rotator and motor assembly 199

57. Cells from an eleven-day-old chick embryo cultivated for sixty-nine days in a roller tube directly on the glass with a fluid medium consisting of 40 per cent horse serum, 40 per cent Earle's salt mixture and 20 per cent chick embryo tissue extract 200

58. Stained sections of fragments of adult rabbit spleen cultivated for four days in a fluid medium containing 50 per cent rabbit serum 205

59. Wooden block for the accommodation of Erlenmeyer flasks being filled with sterile media 206

60. Erlenmeyer flask arranged for preliminary or continuous gassing of culture in which tissue fragments are suspended in thin layer of fluid medium 207

61. Stained sections of fragment of adult rabbit thyroid cultivated for five days in a fluid medium containing 50 per cent rabbit serum (*A* and *B*). Stained sections of fragments of uterus from an ovariectomized mouse cultivated for three days in 125-ml. Erlenmeyer flasks in 3 ml. of fluid medium, approximately one-third of which was rabbit serum (*C* and *D*) 209

62. Stained sections of fragment of human breast tumor after four days' cultivation in a 125-ml. Erlenmeyer flask in 3 ml. of fluid medium, one-third of which was human serum 210

63. Stained sections of fragments of embryo mouse brain after three days' cultivation in a 125-ml. Erlenmeyer

flask in 3 ml. of fluid medium consisting of ox serum ultrafiltrate and Simms' solution (*A* and *B*). Stained sections of fragments from a mixed culture of monkey nerve tissue (olfactory bulbs, sympathetic ganglia and Gasserian ganglion) after three days' cultivation in a Lindbergh flask in a medium containing 25 per cent monkey serum (*C* and *D*) 211

64. Cross-sectional diagram of a watch-glass culture 216

65. Diagram showing the increase in surface area of a colony of fibroblasts belonging to a strain that had been isolated from the skeletal muscle of a fourteen-day chick embryo and cultivated in plasma and Tyrode's solution for thirty-six days 220

66. Curves showing the increase in surface area of colonies of fibroblasts representing two strains isolated simultaneously from the *M. tibialis anterior* of a ten and a twenty-one-day-old chick embryo, and cultivated for fifty-two days (nine passages) in chicken plasma and chick embryo tissue extract 221

67. Diagram showing the increase in surface area of colonies of fibroblasts representing fourteen strains isolated from different parts of a sixteen-day chick embryo and cultivated in a mixture of plasma and embryo tissue extract; from planimetric measurements of the areas attained by the colonies on the sixth day of the seventh passage 222

68. Cumulative curves showing the glucose utilization in roller-bottle cultures containing weighed amounts of chick embryo tissue in plasma and Baker's solution, without added serum 229

69. Diagram showing the changes in ribonucleic acid phosphorus (RNAP) and desoxyribonucleic acid phosphorus (DNAP) in forty-eight small chick-heart explants growing in plasma clots in two roller tubes

for one hundred ninety-two hours with daily renewal of medium containing chick embryo extract and fowl serum 233

70. Stained section of a hanging-drop culture of fibroblasts from bone (*A*). Marginal area of a stained, hanging-drop culture of fibroblasts from skeletal muscle in the twelfth passage (*B*) 237

71. Giant cell in a flask culture of chicken leucocytes infected with avian tubercle bacilli 238

72. Diagrams illustrating the procedure followed in mounting a stained culture grown on a mica coverslip 239

73. Diagrams illustrating the advantages to be gained by using an inverted microscope when working with Carrel culture flasks 261

Methods of
TISSUE CULTURE

TISSUE CULTURE

Chapter I

INTRODUCTION

IT HAS long been known that the death of the organism is not coincident with the death of its parts. Depending upon many factors, the organism may or may not replace the part that has died or been isolated. In certain specific instances the isolated portion may regenerate the entire organism. Failing this, it may regenerate certain or all of its component structures; and, if it does so in surroundings that may be altered at will, accurate observations may be made concerning the specific properties and potencies of the structures in question.

Claude Bernard, in 1878, first pointed out the importance of the internal environment (*milieu intérieur*) in regulating the activities of living tissue. According to his conception,[19] the environment is not only the product of tissue metabolism, but it reacts in turn upon the tissues themselves, and thereby regulates their activity. If, then, an attempt were to be made to study the functional properties of cells, and how these cells affect, or are affected by, their immediate environment, it would be necessary to isolate the different parts in artificial systems in which the influence of the organism could be eliminated. The first step in this direction was taken by Wilhelm Roux in 1885. By isolating the medullary plate of a chick embryo, in a warm saline solution, he actually devised and carried out the first "explantation" experiment on living, organized tissues. Eventually he was able to prove[229] that the closure of the medullary tube is primarily a function of the constituent cells, and not, as some had supposed, the direct effect of mechanical pressure exerted by adjacent structures.

In 1898 Ljunggren[174] made successful transplantations of human skin that had been kept alive for days and even weeks in ascitic fluid. Four years later, Haberlandt[122] published the first of his studies on the isolated vegetative cells of higher plants; and Leo Loeb[177] reported having employed blocks of agar and clotted serum in which small fragments of guinea-pig epidermis were placed for incubation in subcutaneous pockets of another animal. In 1903 Jolly[146] completed an extensive series of observations on the division of amphibian (Triton) leucocytes cultivated for as long as one month in hanging drops. And three years later Beebe and Ewing[15] cultivated an infectious lymphosarcoma of a dog, in the blood of susceptible and of resistant animals. Yet it remained for Harrison[128, 129] to devise a simple technique that would permit explanted parts to continue to grow and develop outside of the organism. In a sense Harrison's experiments were the logical development of the microsurgical procedures of elimination and transplantation that had been introduced to the method of experimental embryology by Roux* and Born†; for if it were possible to transplant living tissues and allow them to develop further in new surroundings within the body, it should also be possible to find some external culture medium in which they would likewise grow.

Harrison published his first observations on the living, developing nerve fiber in 1907. The ingenious experiments constituting the basis of this report[128] not only gave ocular proof of the histogenesis of nerve outgrowth; it also provided a striking demonstration of the usefulness of tissue culture in studying the structural elements of the higher organisms in relation to their environment. Previously, in an attempt to prove the contention of His and Ramón y Cajal that the nerve fiber is formed as the outgrowth of a single cell, Harrison had transplanted fragments of the nervous system of amphibian larvae to other parts of the developing embryo. He found that nerve fibers always developed in surroundings composed of living, organized tissue.

* Roux, W. *Z. Biol.*, 1885, *21*, 411.

† Born, G. *Arch. Entwcklngsmechn. Organ.*, 1896-97, *4*, 349, 517.

This situation was unfortunate. It introduced the possibility that the latter might contribute organized material to the nerve elements. But it was even more unfortunate because one of the main opposing theories to be tested presupposed the existence of some sort of primitive structures, or protoplasmic bridges, that pervaded all parts of the embryo and later gave rise to nerve fibers. Hence, it became necessary to isolate the nerve centers from the body and to determine their power to form nerve fibers in a medium completely devoid of cell structure. To achieve this end, Harrison removed small fragments of undifferentiated embryonic tissue from the walls of the neural tube and suspended them in hanging drops of clotted lymph from the adult animal. His hopes were fully realized, for not only did the cultures show active growth, but with aseptic precautions they could be kept in good condition for several weeks. It now remained only to make the decisive observations. These indicated very clearly that the nerve fiber was actually formed by the spinning of a thread of neuroblastic protoplasm into a filament by means of the free end of the process. Thus, the cell alone was shown to be concerned in the formation of the axone.

For the development of the more precise techniques from these early procedures of Harrison, biologists are mainly indebted to Carrel, who promptly saw in them the possible means of realizing to a fuller extent than ever before the exacting precepts laid down by Claude Bernard. Carrel had been ardently engaged in applying the principles of surgery to the physiological study of mammalian organ systems. His extensive experience in this field had thoroughly convinced him that the greatest hope of arriving at a clearer understanding of the laws of growth and organization rested in the study of such structural units as could be isolated from the organism and cultivated in media of known composition. Thus, with the gradual improvement in techniques and the progressive elaboration of suitable media, it soon became possible to derive thousands of cultures in pedigree fashion from the progeny of one. A strain of connective-tissue cells from the chick that served as the test material for much of Carrel's work[34, 41] was kept in a state of active multiplication for thirty-four years (Fig. 2).* This and other cell strains developed in many laboratories have provided an abundance of uniform material for physiological studies of every sort and description, and as a result a great deal has been learned concerning the true nature of cells and their specific requirements. Cells and tissue fragments isolated from almost any part of the body and placed in suitable culture containers can be maintained under conditions that are amazingly constant, and may be observed at will under the highest powers of the microscope.

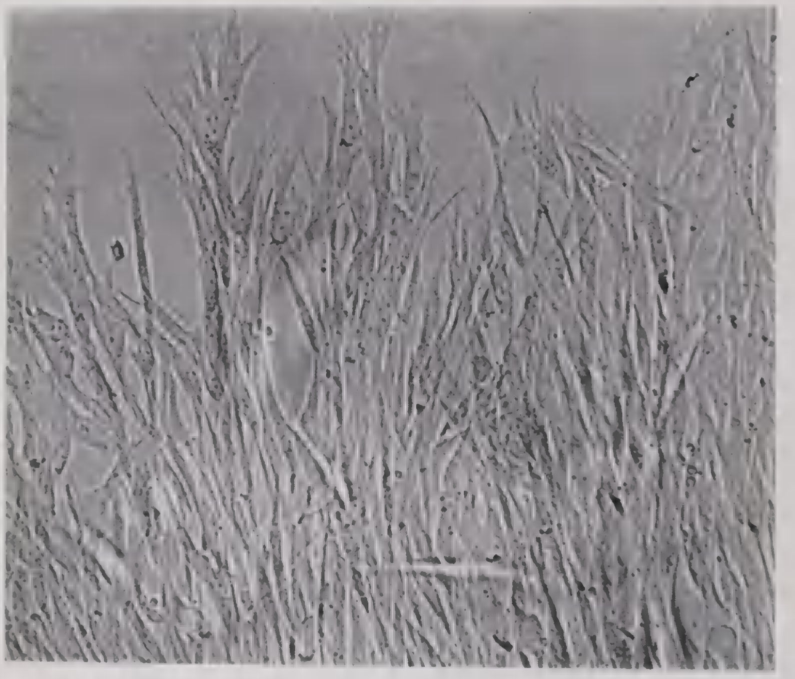

FIG. 2. Marginal area of culture of Carrel's strain of chick embryo spindle cells (fibroblasts) after strain had been propagated for twenty-six years. (A. H. Ebeling, unpublished data, 1938.) ×230.

Among the various animal cell types that have been cultivated for longer or shorter periods *in vitro* are: epithelial and epithelial-like cells, fibroblast-like spindle cells and other mesenchymal elements from the connective tissue, cells from cartilage and bone, nerve cells, muscle cells, reticular cells from bone marrow, lymph nodes and spleen, cells from the gonads, endometrium, trophoblast and placenta, and leucocytes from the blood, the majority of which may be derived either from the embryo or the adult. In addition, the techniques have

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* Ebeling, A. H. Personal communication.

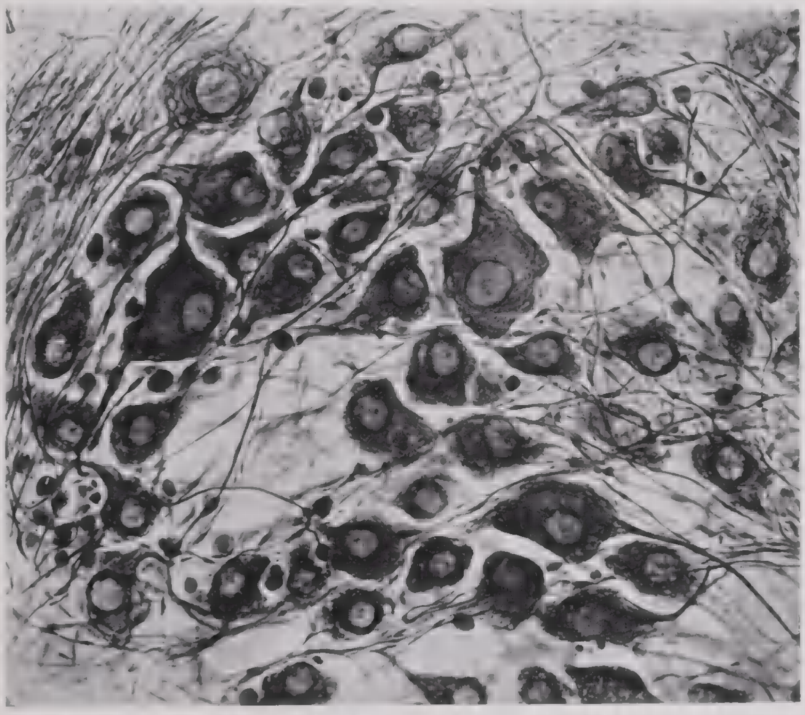

FIG. 3. Spinal ganglion cells from an eleven-day chick embryo; fixed and stained after five days' cultivation in equal parts of chicken plasma and embryo tissue extract. (G. Levi and H. Meyer.[161]) ×300.

been used to advantage for the continued maintenance, in a living state, of explanted fragments of complex tissue structures, consisting of a variety of cell types; parts of mature organs, in which the component tissue structures have already achieved definite form; developing structures, in which the growing complexity may be studied under controlled conditions; and malignant cells from many sources. Although the tissues are ordinarily taken either at biopsy or soon after the death of the individual, cultures of certain cell types may be

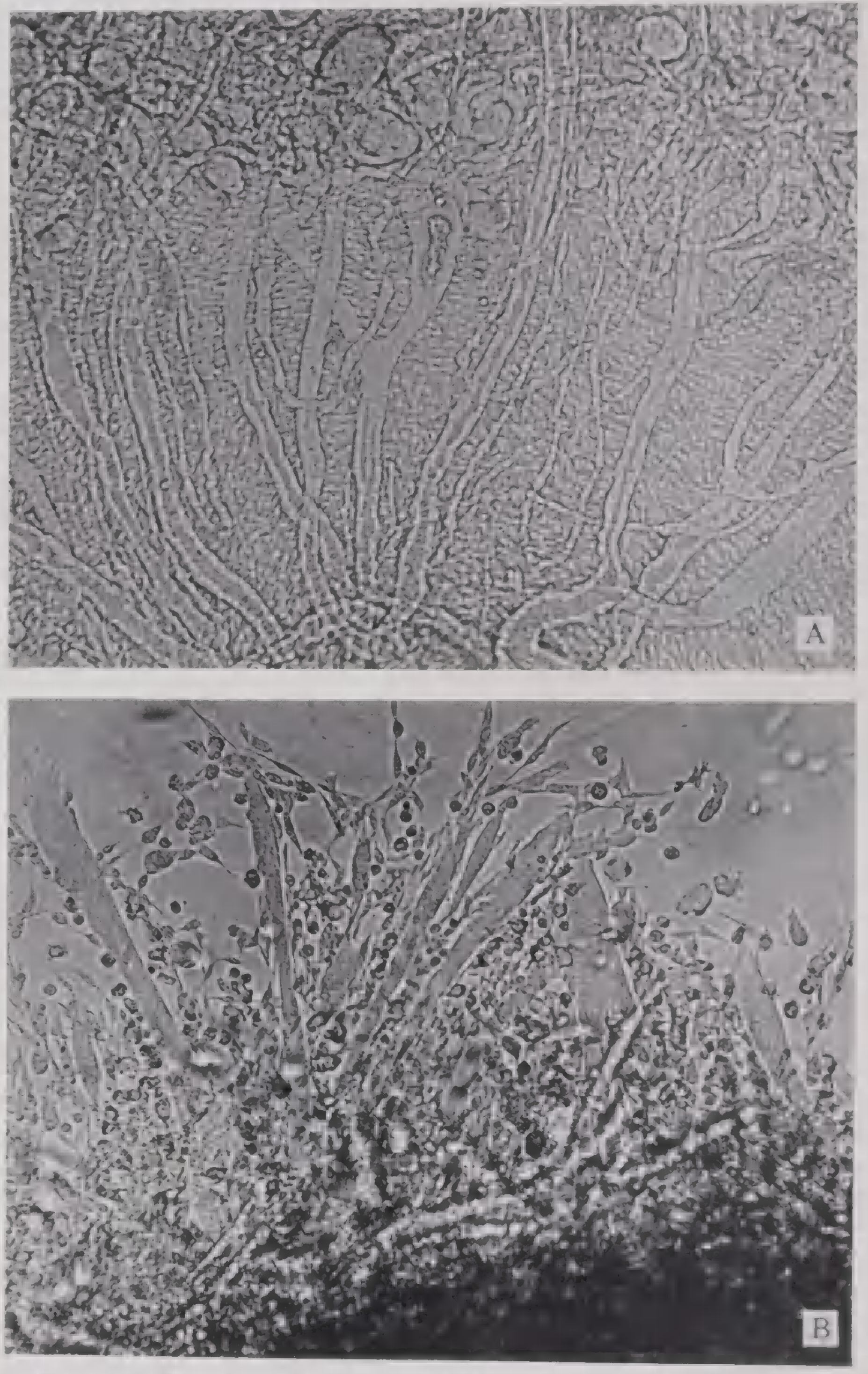

FIG. 4. *A.* Fragment of embryo rat kidney after nine days' cultivation in chicken plasma and rat serum. ×115.

B. Culture of skeletal muscle from an embryo rat. Note the development of several well-defined muscle buds. ×115.

initiated from cold storage material (at 4°C., for example) many days after death.[127]

In addition to the developments made in Carrel's laboratory, the original techniques of Harrison have been elaborated and improved along somewhat different lines by other workers, notably by Lewis and Lewis, Maximow, Levi, Champy, Braus, Brachet and Strangeways. The combined effect of all of these efforts has been the gradual development of a system of procedures that has been used to advantage in almost every field of experimental biology and medicine, particularly in cytology, histology, embryology, cell physiology, cell pathology, bacteriology and immunology, and in the study of tumors and viruses. It has been possible, for example, to study: the structural and functional characteristics of the various cell types that comprise the individual; the formation, function and diseases of the blood; the nature of the various forms of energy-producing reactions and other chemical changes occurring in the culture system; the conditions necessary for tissue grafting; the nature of cell movement; the process of cell division in all its phases; the special potencies of undifferentiated embryonic regions and organ rudiments, at various stages of development; the nature of the Golgi elements, the mitochondria and other cytoplasmic structures; the elaboration of hormones and other secretion products; the specific action of drugs, antiseptics, antibiotics and other chemical substances on various cell types and organ structures; the effect of irradiation, temperature and other physical forces on cells; the reaction of cells of various types to bacteria and bacterial products; the conditions necessary for the propagation of viruses; the food requirements of the various cell types and the nature of substances required in chemically defined nutritive media; the site of, and the conditions necessary to, the formation of various types of immune substances; the elaboration of cytotoxins and the nature of hypersensitivity; the similarities and differences between tumor cells and normal cells of similar origin; the transformation of normal cells to malignant cells; the distribution and utilization of radioactive tracer substances incorporated in the medium; and the microcytology of cells by means of the electron microscope.

Because they are easy to cultivate, the common connective-tissue cells have been studied more systematically than any of the others. In 1912 Carrel[34] succeeded in isolating a strain of these cells from the heart of an embryo chick. This strain (Fig. 2) lived and multiplied for over thirty-four years and retained its original characteristics throughout the entire period of cultivation. For convenience, the cells were called "fibroblasts." In the course of time, however, they came to be identified by their living properties. Their rate and manner of locomotion, their food requirements, their rate of proliferation and their morphological appearance under given environmental conditions, their action on the medium, and their reaction to vital dyes, were studied and recorded.[41, 47] Then, when cells of a similar

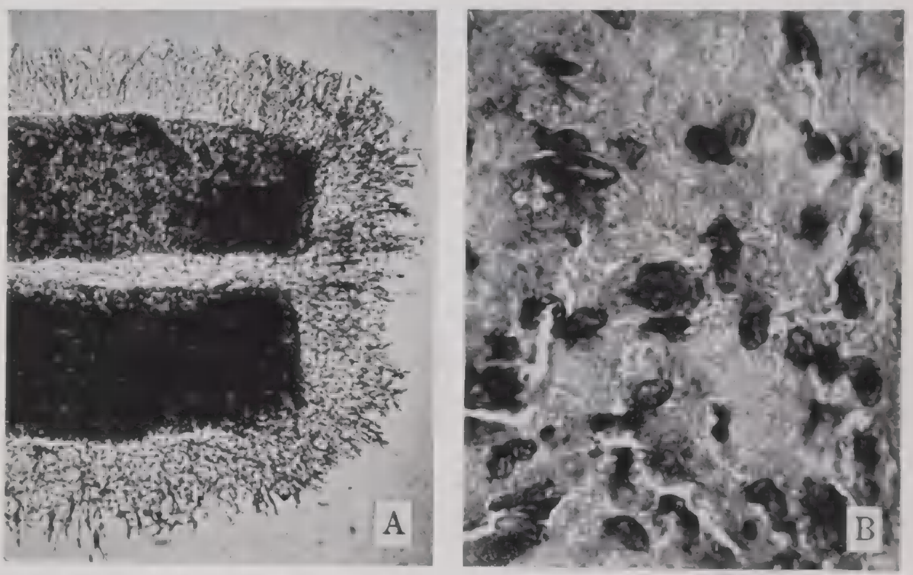

FIG. 5. *A*. Fringe of new cells surrounding fragments of tissue excised from a culture of chick embryo breast muscle that had been embedded in a plasma coagulum and treated with adult chicken serum for one year before being transferred to a fresh flask.

B. Section through center of a tissue fragment that had been treated with adult chicken serum for one year in a single flask. Fixed in Zenker-formol and stained according to Masson's trichrome procedure. (R. C. Parker.[209])

nature were obtained from other parts of the organism, they too were called fibroblasts.

Now, it is known that fibroblast-like cells, as a group, comprise many cell races.[205] Each race manifests certain specific functional properties when cultivated *in vitro*. These properties, which are remarkably persistent, differ according to the age of the individual and also according to the particular organ or tissue from which the strain is derived.[208] They are displayed in the relative abilities of the various races to live and multiply in a given environment and to reproduce demonstrable changes in the physicochemical nature of that environment. It follows, then, that the characteristics manifested by a given cell strain in a particular medium reflect not only the nature of that medium, but also the physiological state, at the moment of isolation, of the part of the organism from which it is taken. When fibroblast-like cells from connective tissue are grown in cultures, they develop a loose meshwork in which the cells,

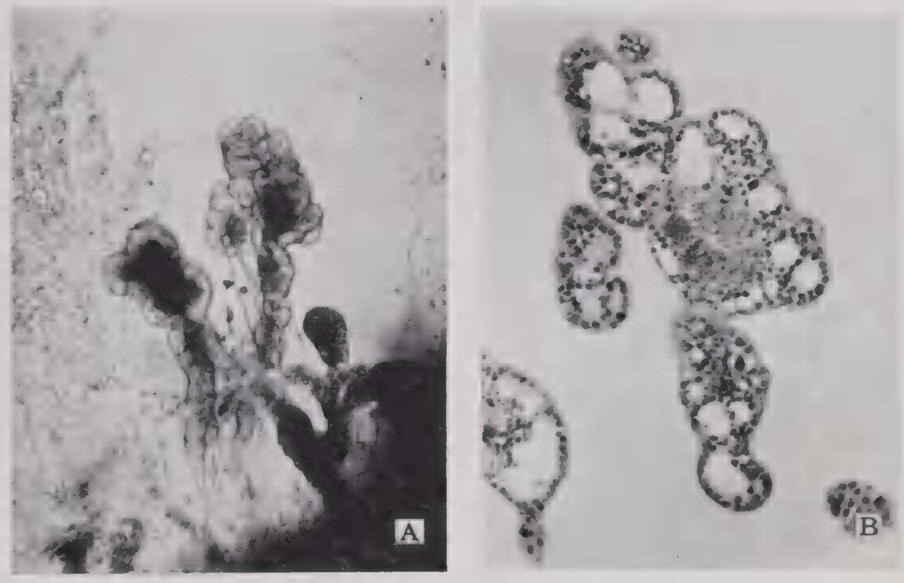

FIG. 6. *A*. Tubules from the marginal area of a culture of mouse carcinoma (E-0771) after six months in a single flask treated regularly with 40 per cent Earle's salt mixture, 40 per cent horse serum and 20 per cent chick embryo tissue extract. ×41.

B. Stained section through the central cluster of tubules shown in *A*. ×100.

though intimately associated with one another, may be in several layers. Epithelial cells, on the other hand, migrate out from the tissue explant to form more or less continuous sheets in which the cells tend to arrange themselves in a mosaic. Strains of these and other cell types may be carried indefinitely in cultures and become remarkably uniform in character if they are maintained in a state of active multiplication.

Under these conditions, which are so advantageous for the propagation of cell strains for use as biological test material, most types of cells tend to lose their more specialized functions and assume the appearance of fibroblast-like spindle cells, amoeboid-like macrophages with undulating membranes, or indifferent epithelial-like cells. If, however, the cultures are allowed to remain in the same container for long periods (months, for example), and are regularly supplied with a medium in which the growth-stimulating substances are reduced to a minimum, cells that were once quite similar in appearance tend to develop striking dissimilarities and proceed to display still other properties commonly associated with them in the organism.[209] Thus, by suppressing cell multiplication, malignant mammary gland epithelium may form complex tubules (Fig. 6), iris epithelium may form pigment,[74] thyroid epithelium may form colloid,[84] and connective tissue cells may form collagenous fibers.[184] Also, under certain conditions, fibroblast-like spindle cells take on the appearance of macrophages, and macrophages become transformed into spindle cells. Bloom[24] considers all these various changes to be reversible reactions and prefers to call them *modulations* as distinct from more permanent changes that make for greater specialization in actual and potential functions. But even here it is generally agreed that it is exceedingly difficult to decide whether the level of specialization attained by a particular cell is permanent or temporary, reversible or irreversible. As Harrison[131] points out, "There is no way of finding out with certainty whether the particular quality which a cell seems to have is finally fixed, for there always may be new conditions, not yet tested, under which other potencies might be revealed."

Additional information having to do with the growth, metabolism and nutritive requirements of tissue cells cultivated *in vitro* will be found in Chapters VII, VIII and XV; and interesting experiments on blood leucocytes are described in Chapter XI.

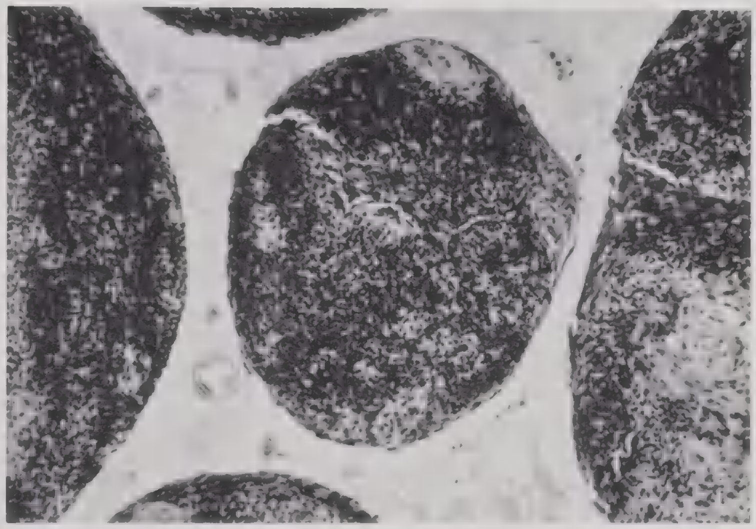

FIG. 7. Stained section of fragments of adult chicken spleen after twenty days' cultivation in a fluid medium consisting of 50 per cent rabbit serum in Tyrode's solution containing extra bicarbonate. The medium was renewed at five-day intervals. × 110.

In an effort to determine some of the underlying factors that make for greater specialization, several investigators have employed the method of tissue culture as a means of studying the further development of such complex structures and organ rudiments as the metanephros, undifferentiated limb buds, the knee-joint, sternum and gonads, eye and ear rudiments and isolated tooth germs.[88] Some of the structures show advanced histological development *in vitro*, but little or no anatomical development. Skeletal rudiments, however, continue their anatomical development in a comparatively normal way. Thus, when Fell and Robison[89] removed the femur rudiment from a five-and-one-half-day-old chick embryo and grew it in culture, it increased to more than three times its original length, the condyles, head and trochanter became dis-

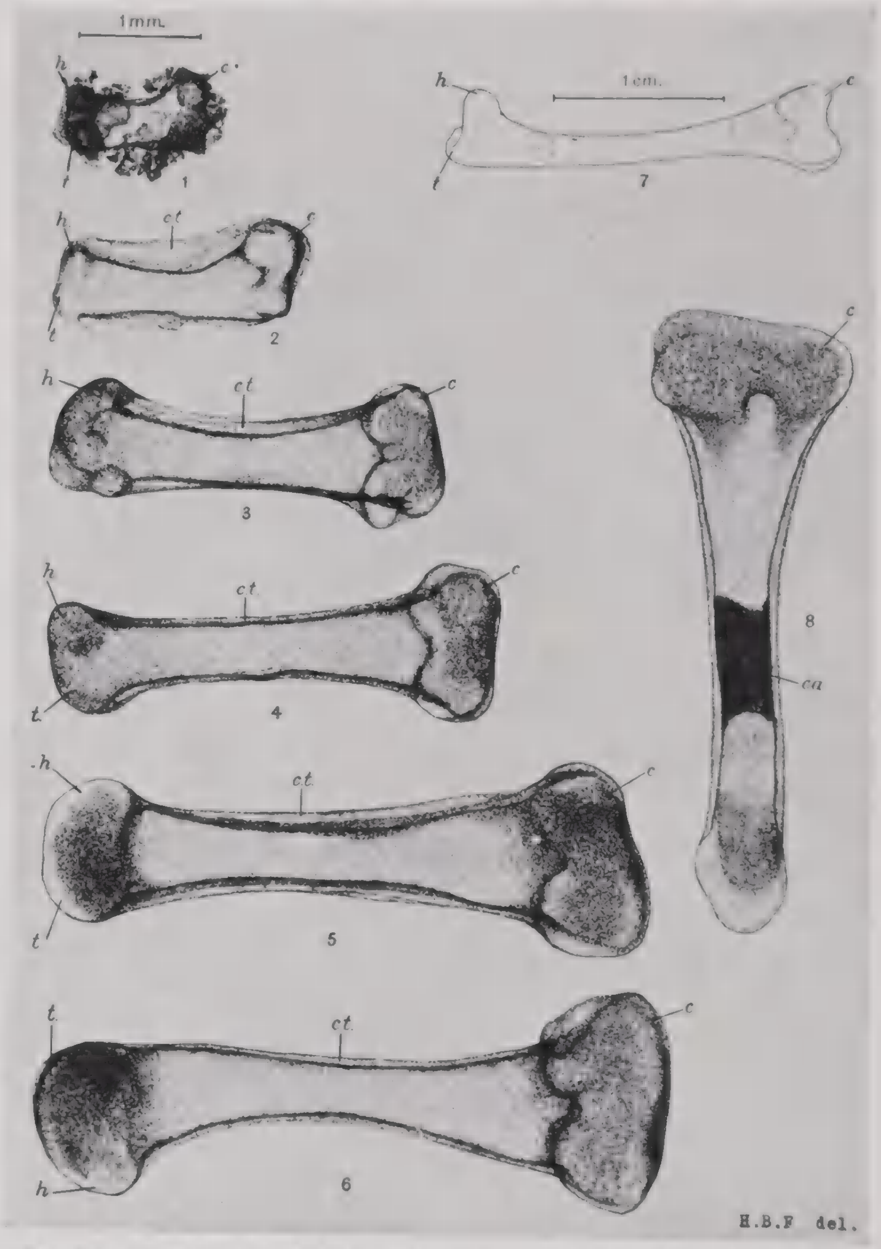

Fig. 8. Development of chick embryo femora cultivated on the surface of a clot consisting of equal parts of plasma and embryo tissue extract: (1) normal five-and-one-half day femur as dissected for explantation; (2) after three days' cultivation; (3) after nine days; (4) after fifteen days; (5) after twenty-one days; (6) after twenty-seven days; (7) normal femur from twenty-one day embryo; (8) five and one-half day femur after twenty-seven days stained by von Kossar's silver nitrate method. (H. B. Fell and R. Robison.[89])

tinct, an epiphysis developed, and a sheath of periosteal bone was formed around the shaft (Fig. 8). And by correlating the histogenesis and special chemical requirements of isolated fragments, these authors[90] added much to our knowledge of the development of bone and cartilage. Gaillard[112] has employed what he describes as a "dynamic medium" in the study of developing structures in tissue culture. According to his procedure, the fluid medium used on plasma cultures in flasks is replaced every two or three days with a fluid containing chick embryo extract prepared from embryos that are two or three days older than those used in the extract incorporated in the previous medium. By this means Gaillard was able, for example, to obtain an unusually rapid differentiation of developing bone structures (see p. 109).

W. H. Lewis,[168] Pincus[213] and their collaborators have made cinematographic records of the maturation and early cleavage stages of cultivated rabbit ova. And Waddington and his coworkers, by repeating many of Spemann's amphibian experiments on explanted chick blastoderms cultivated on plasma clots, have contributed new facts concerning the developmental mechanics of warm-blooded animals. For example, any part of the avian primitive streak, or even the whole of it, can be regenerated after extirpation.[256] Furthermore, the endodermal layer of the primitive streak has an organizing function that resembles the amphibian organizer. When grafts of the primitive streak, cleaned of ectoderm, are inserted between the ectoderm and endoderm of an explanted blastoderm, a second embryo is formed in the overlying ectoderm.[257] Even rabbit primitive streak will induce a second embryonic axis when grafted into fowl blastoderms grown in watch-glass cultures.[259] Moreover, these influences can be exerted by dead cells, and by cells coagulated by heat.[258] It has been shown by Holtfreter,[139] however, that unspecific stimuli that cause a temporary sublethal state of cytolysis may determine certain cells to become neural instead of epidermal. Once induced, the neural tissue has the capacity of transmitting its histological character to other competent tissues. Holtfreter has extended this concept to the interpretation of inductions obtained with dead tissues, toxic grafts and normal inductors, and has drawn interesting parallels between the phenomena of neural induction, parthenogenesis and malignancy (see p. 26), the understanding of any one of which may provide the explanation for the others.

An interesting application of tissue culture in the endocrine field involves the preparation of homologous grafts for transplantation. In 1934 Stone, Owings and Gey[245] applied the method with some measure of success to the treatment of human thyroid and parathyroid deficiencies. The tissues were cultivated in the recipient's plasma and serum for about two weeks prior to transplantation, and were eventually implanted in the axilla. More recently Gaillard[112] cultivated fragments of human parathyroid from newborn infants for about fourteen days and grafted the explants into patients suffering from postoperative tetany. The glands were cut into small fragments that were cultivated separately, by the coverslip method, in a drop of medium consisting of 20 per cent human plasma, 8 per cent human serum, 4 per cent chick embryo extract, and Gey's balanced salt solution. Both the plasma and serum were obtained from the individuals who were to be the eventual recipients of the grafts, and the extract that was used was barely enough to induce coagulation of the plasma. After twelve hours' incubation, the fibrin of the coagulum became digested and the fragments floated in the fluid; the medium was changed every three or four days. Each of fifteen patients received from nine to fifteen fragments that were grafted, according to the method of Stone, Owings and Gey,[245] into the perivascular lymph space of one of the axillary veins. The grafts did not take in any of the six recipients who were over thirty years of age, but of the younger recipients, six responded positively to the grafts, two showed temporary improvement, and one showed no improvement. It has long been the hope of clinicians that

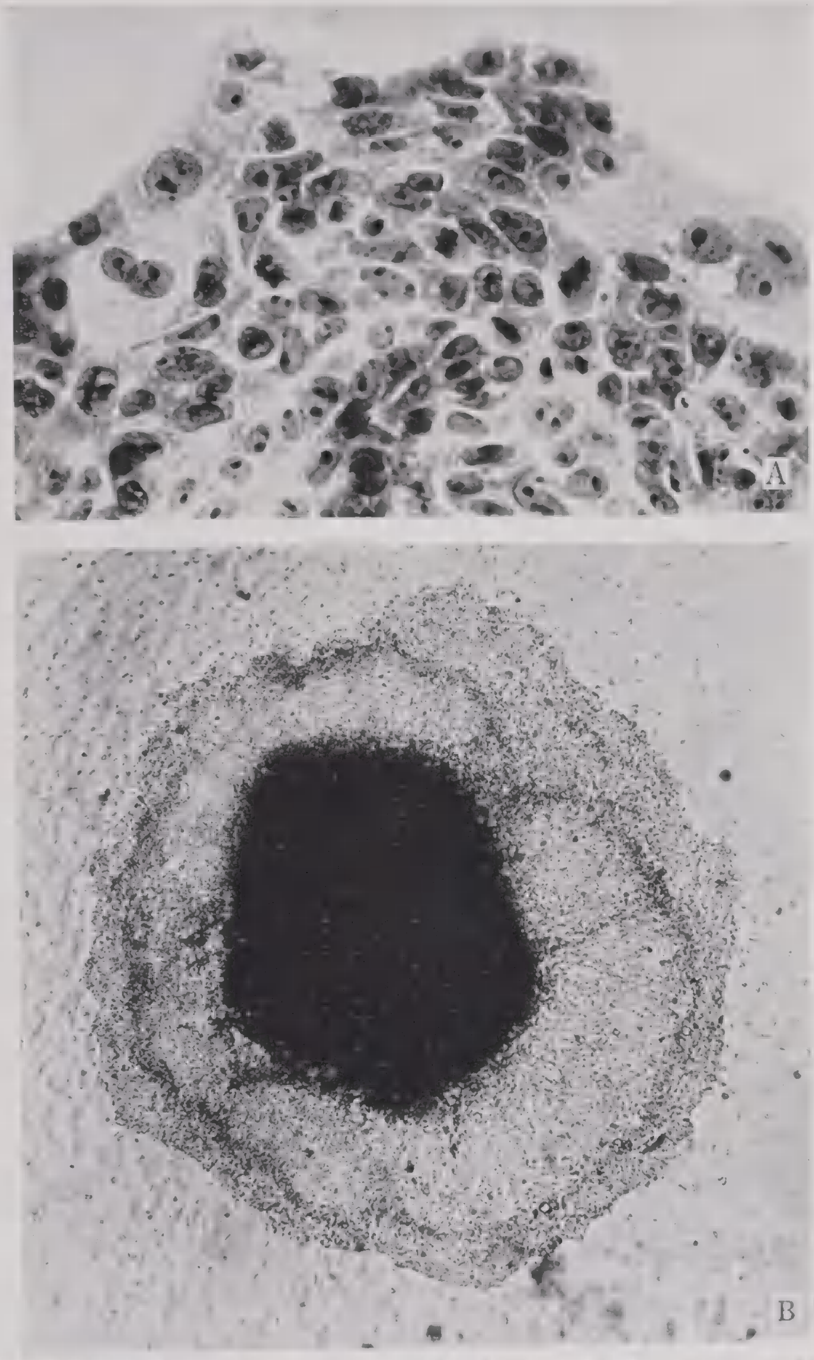

FIG. 9. *A.* Marginal area of a stained culture of Fischer's strain of Ehrlich mouse carcinoma in the two hundred sixty-second passage. (A. Fischer.[97])

B. A four-day-old culture of spontaneous adenoma from the mouse. (L. Santesson.[235]) × 42.

this method of treating certain endocrine deficiencies might one day become practical enough to be of general use.

Although the behavior of a tumor is but a secondary manifestation of the activity of its constituent cells, certain fundamental characteristics of these cells remain hidden as long as they are parts of the whole. It is possible, however, to study the functional and structural properties of tumor cells by isolating them in cultures and by comparing them with normal cells of similar origin. In this manner a small group of investigators has uncovered many interesting facts that contribute in large measure to the present conception of the malignant cell.

One of the first problems was to determine whether the cells retained their malignancy *in vitro*. As early as 1911 Carrel and Burrows[45] had cultivated the Rous chicken sarcoma, the Ehrlich and the Jensen rat sarcomas, the Flexner-Jobling rat carcinoma, and an adenocarcinoma of a dog, while Volpino[255] had cultivated an adenocarcinoma of the mouse, and Lambert and Hanes[153] had succeeded in carrying transplantable sarcomas of both rats and mice. Although the technical difficulties were great it was possible to keep the cells alive *in vitro* and to determine, even then, that tumor cells were not easily distinguishable from other cell types.[45] Furthermore, both the Rous sarcoma cultures of Carrel and Burrows[44] and the mouse adenocarcinoma of Volpino produced tumors when grafted back into the animals.

It was almost fifteen years later, however, before a systematic attempt was made to cultivate strains of tumor cells for long periods. Once this was accomplished it was found that they retained their malignancy indefinitely, regardless of the length of time they were carried *in vitro*. Fischer carried the Rous chicken sarcoma in cultures for three and one-half years[97] and the Ehrlich adenocarcinoma of the mouse (Fig. 9) was cultivated for twelve years[108] in a medium completely devoid of mouse material. Similar experiments were made by Lewis and his associates, who cultivated malignant cells from six different rat sarcomas for periods ranging from two to four years.[165, 166]

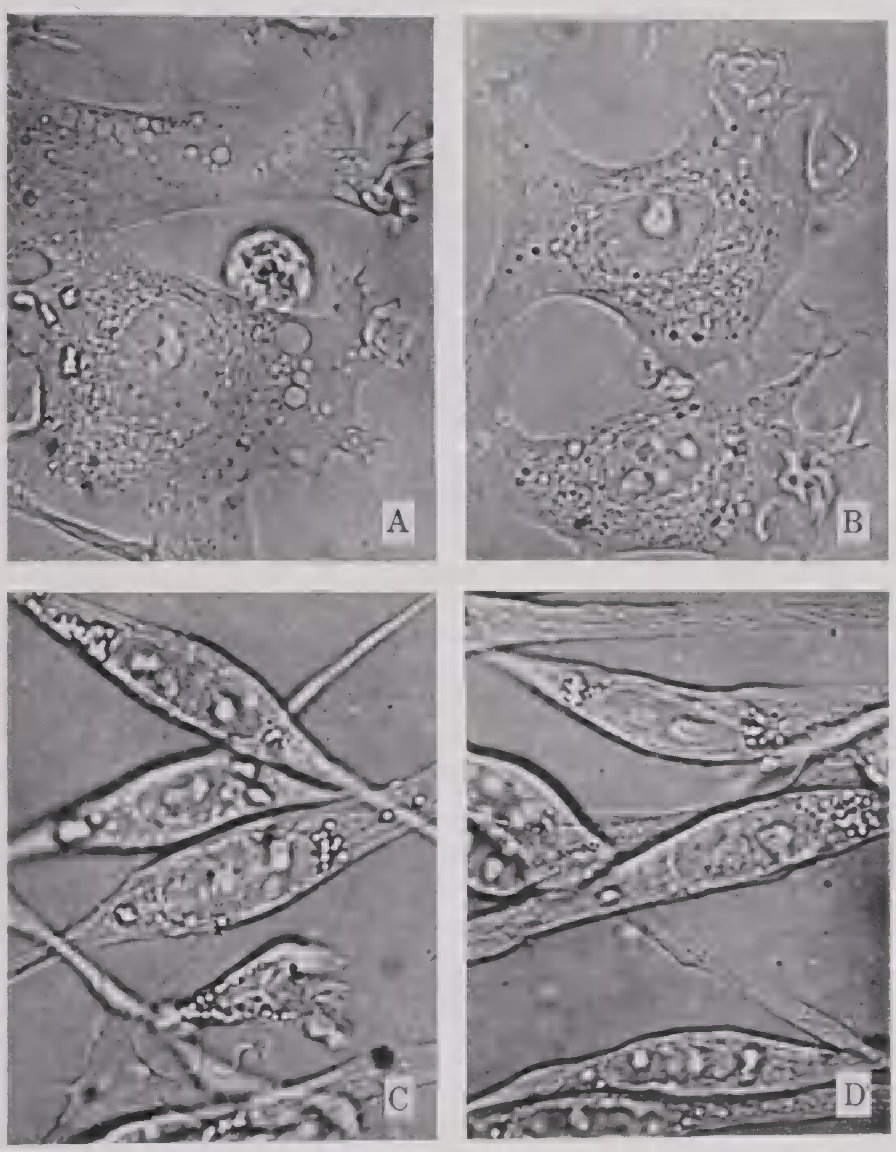

FIG. 10. *A*. Malignant cells from dibenzanthracene mouse tumor C37 removed from an animal after three generations and cultivated for one day in a hanging drop. (Compare Fig. 10*B*.) × 1100.

B. Cells from a culture of the tumor shown in *A*, but removed from an animal after twenty-five generations. The cells have retained their peculiar morphological features through twenty-two animal passages. (W. H. Lewis, unpublished data.) × 1100.

C. Malignant cells from mouse tumor PHS 1 (produced by dibenzanthracene) removed from an animal after twenty-two generations and cultivated for one day in a hanging drop. (Compare Fig. 10*D*.) × 1100.

D. Cells from a culture of the tumor shown in *C*, but removed from an animal after thirty-seven generations. The cells have retained their peculiar morphological features through fifteen animal passages. (W. H. Lewis.[166]) × 1100.

Here again, the various strains retained their essential cultural and cytological characteristics, and their malignancy, over the entire period of cultivation. In addition, Lewis had studied the malignant cells from a large number of mouse tumors produced by 1:2:5:6-dibenzanthracene. From time to time tissue explants were removed from the serially transplanted tumors and examined in cultures. In every instance, however, the malignant cells retained their essential characteristics despite many passages through mice (Fig. 10). It would seem then, that malignancy is a relatively permanent characteristic located in the cells themselves.

In their mode of colony formation, malignant cells are often indistinguishable from normal cells. Sarcomatous fibroblasts resemble normal fibroblasts; carcinomatous cells grow in sheets that resemble normal epithelium.

Again, the residual growth energy and the rate of proliferation of normal and of sarcomatous fibroblasts from the same type of tissue are remarkably similar. When colonies of both cell types are placed in a medium devoid of readily available foodstuffs, the curves representing the rate of migration of the cells and the duration of their survival are often identical.[49, 50] When both types of cells are placed in a nutrient medium, they may proliferate at the same rate. It follows, therefore, that malignant cells neither accumulate more reserves nor is their velocity of growth necessarily greater than that of normal cells. In fact, Fischer and Laser have shown that carcinomatous epithelium from the mouse (Fig. 9) grows more slowly *in vitro* than does normal mouse epithelium.[109] It may be that the increased rate of growth observed in the body is not a primary characteristic of malignancy but rather a secondary phenomenon resulting from the utilization of break-down products set free within the tumor itself.

Although there may seem to be certain differences in the food requirements of normal and of malignant cells, these differences do not appear to be greater than those existing between various types of normal cells or even between various strains of the same general cell type. But until a chemically defined synthetic medium has been devised (see Chap. VIII), it will not be possible to determine with any degree of assurance whether any marked nutritional differences exist between the two cell types. Malignant cells cultivated in natural body fluids and extracts are not especially fastidious in their dietary requirements; but neither are normal cells. Both normal and malignant cells from a mouse, a rat or a human may be cultivated indefinitely in materials derived from other species; and many animal tumor strains are known to retain their malignancy under these conditions for years. Still, there is as yet no means of knowing whether malignant cells may not possess certain means of adjusting themselves to an environment that is inappropriate for the continuous multiplication of normal cells. It is perhaps possible that explanted tumor tissues behave like explanted normal tissues only because it is unnecessary for tumor cells, living in the artificial environment of a tissue culture, to fight for survival. So long as they continue to be fed and cared for outside the body, the real distinguishing characteristics of tumor cells may remain latent.

From time to time various authors have expressed the opinion that malignant cells are characterized by abnormal cell divisions, but while this is undoubtedly true of certain tumors, it is by no means a general rule.[163, 165, 166] Attention has also been given to the prevalence of dying cells in many tumors, a characteristic manifested both *in vivo* and *in vitro.* It has even been suggested by Lewis and Strong[163] that the process by which the organism rids itself of this great mass of dead material may also be involved in the mechanism that controls the cancer process.

It is generally accepted that virus-like agents are intimately associated with certain fowl tumors, of which the Rous chicken sarcoma is the best-known example, with the Shope rabbit papilloma and with the milk-factor tumors occurring in certain strains of mice. Quite recently Porter and his associates[217] have developed special procedures for examining tissue-culture cells by means of the electron microscope (Fig. 11) and have made a careful study of the microcytology of tumor cells from various sources. In cells from tumors known to be induced by cell-free materials, they found virus-like particles, whereas no viruses were found in cells from tumors of unknown etiology.[56, 220] In these latter cells, however, they observed the presence of great numbers of dense endoplasmic granules of a sort also found in

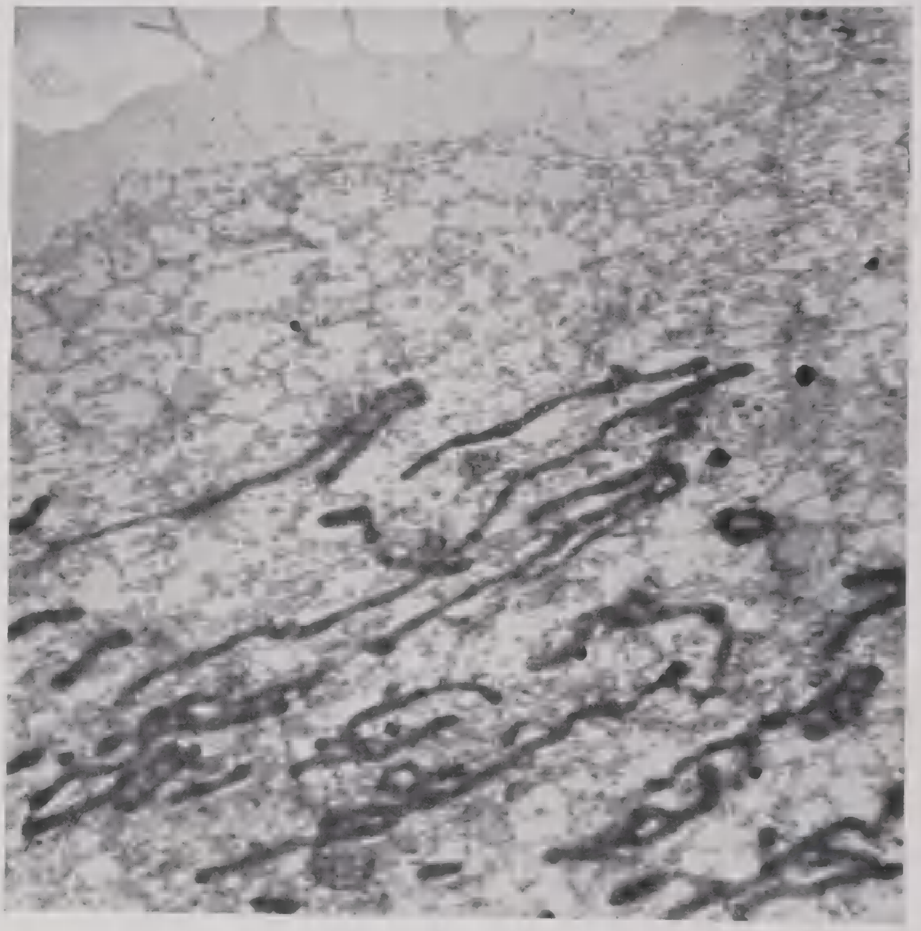

FIG. 11. Electron micrograph of the margin of a cell from a culture of rat fibroblasts. Mitochondria are evident and show some internal structure and a surrounding sheath. Vesicular bodies are seen in the endoplasm. The marginal area of the cell is free of granules. Osmic acid fixation. (K. R. Porter, courtesy of the W. B. Saunders Company.) ×5600.

normal cells, but in lesser abundance.[219] In certain respects, these endoplasmic granules had the appearance of multiplying units.

One striking feature that sets tumor tissues apart from normal tissues is the relative ease with which the former may be transplanted from one animal to another. As long as the cancer process remains obscure, the explanation of this feature of malignancy will probably remain hidden. Once it is understood, however, biologists should be in a better position to prepare normal tissues for homoplastic and heteroplastic transplantation, an accomplishment that would be almost as great a boon to experimental biology and medicine as the solution of the cancer problem itself.

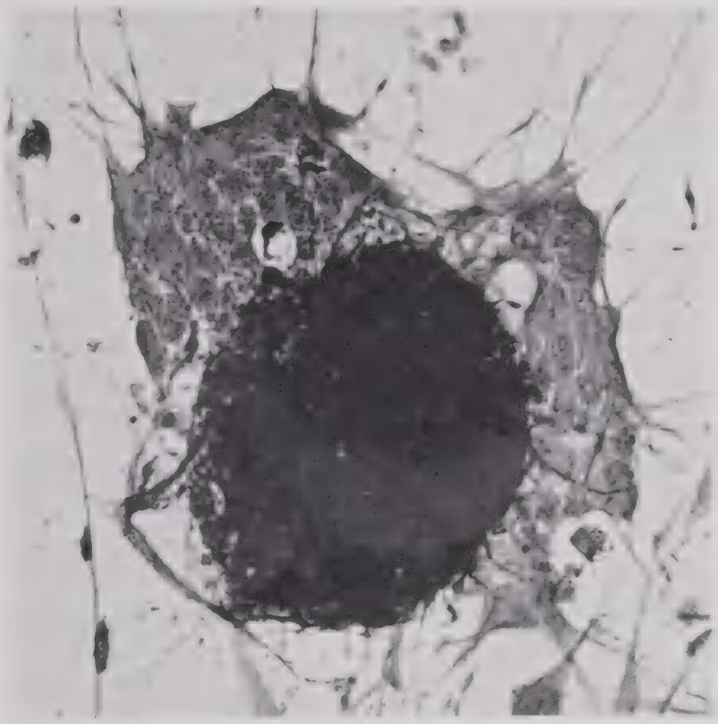

FIG. 12. Seventeen-day-old double-coverslip culture of human sympathicoblastoma (or neuroblastoma) growing as sheet-like epithelium with neurites and fibroblasts. Bodian's silver stain. (M. R. Murray and A. P. Stout.[202]) ×250.

The tissue culture techniques have also been used successfully in the differential diagnosis of certain tumors, particularly those of neural or mesoblastic origin. The main advantage of the techniques rests in the ease with which it is possible to free living cells from the compact tumor masses and to study their histogenic relationships. Thus, Chlopin[55] and Murray and Stout[199, 200] were able to determine that the neurilemmoma is a neoplastic growth of Schwann cells rather than one of perineurial cells, a point that had formerly been disputed by pathologists. This was accomplished by cultivating normal peripheral nerves, in order to distinguish between Schwann cells and the cells of the endo- and perineurium, and by comparing the normal cultures with the outgrowth from neurilemmomas. Tissue culture was also used by Murray and Stout[202] in the differential diagnosis of sympathicoblastoma, or neuroblastoma (Fig. 12). This tumor is found more frequently in children than in adults and can be confused with Ewing's tumor, with tumors of the lymphosarcoma group, or with small-celled carcinomas. In tissue culture, however, the neoplasm is found to consist of embryonal sympathetic nerve cells and it produces typical neuroblasts and a primitive type of neuroepithelium from which neurites develop. Under favorable conditions, a diagnosis can be made within twenty-four hours.

The tissue-culture method of diagnosing tumors and determining cell origins has also been used by other investigators, notably by Russell and Bland,[230, 23] Hengstmann,[135] Coman,[59] Hoster et al.,[140] Sano and Smith,[234] and Grand.[117] In general it may be said that in certain instances the use of tissue culture as a means of diagnosing tumors may (1) be quicker and more reliable than the routine section method (e.g., sympathicoblastoma[202]), (2) provide a useful supplement to the clinical and pathological findings (e.g., neurilemmoma,[198, 200] Hodgkin's disease[117]), (3) serve to distinguish between several possible diagnoses (e.g., liposarcoma,[201] rhabdomyosarcoma[249] and fibrosarcoma or myxoma), or (4) be of no obvious benefit.

Tissue culture also provides a means of transforming normal cells to malignant cells by the direct action of carcinogenic agents. Fischer,[96] Laser,[155] Bisceglie[20] and des Ligneris[169] have each reported cancerization in cultures of embryonic tissues by using tar, arsenic acid, x-rays, plasma from animals previously treated with tar, and 1:2:4:6-dibenzanthracene. In 1943, however, a much more elaborate experiment was reported by Earle,[77, 79] who exposed strains of normal mouse cells grown in heterologous media to 20-methylcholanthrene for periods ranging from six to 406 days. The strains all arose from a small clump of subcutaneous connective and adipose tissue taken from a one-hundred-day-old C3H mouse. Following the treatment with methylcholanthrene (1 gamma per ml. of medium), the strains were cultivated for long periods without the carcinogen, some for several years (see p. 26). But over a period of from twenty-five to ninety days after the first addition of carcinogen to the cultures, several of the strains developed an altered morphology that was most striking. The cells lost their fibroblast-like appearance, became rounded and granular, and showed a tendency to grow in epithelial-like sheets; in addition, many large giant cells developed. Upon injection back into C3H mice, several of the altered strains consistently produced tumors (sarcomas) all of which were readily transplantable and some of which metastasized. Eventually, however, certain of the control strains also developed an altered morphology and produced tumors upon injection. Although the control strains had had no known contact with the carcinogen, Earle felt that the possibility of trace contaminations could not be entirely ruled out. It is also interesting to note that the highest percentage of tumors were obtained from control cultures even though they showed less morphological change than did some of the treated cultures. More recently, Firor and Gey[93] have reported spontaneous transformations of a similar nature in cultures of normal rat tissues.

In the author's laboratory, an effort was made to repeat* the experiments of Earle on a much smaller scale and with but one major change in the basic plan, namely, that the tissues were derived from mice belonging to a strain of albino (Swiss) mice that had been inbred by brother-sister matings for at least twenty-one generations.† These mice are remarkably free from

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* Parker, R. C. Unpublished experiments.

† The strain of mice was supplied by Dr. C. J. Lynch, of The Rockefeller Institute for Medical Research, New York.

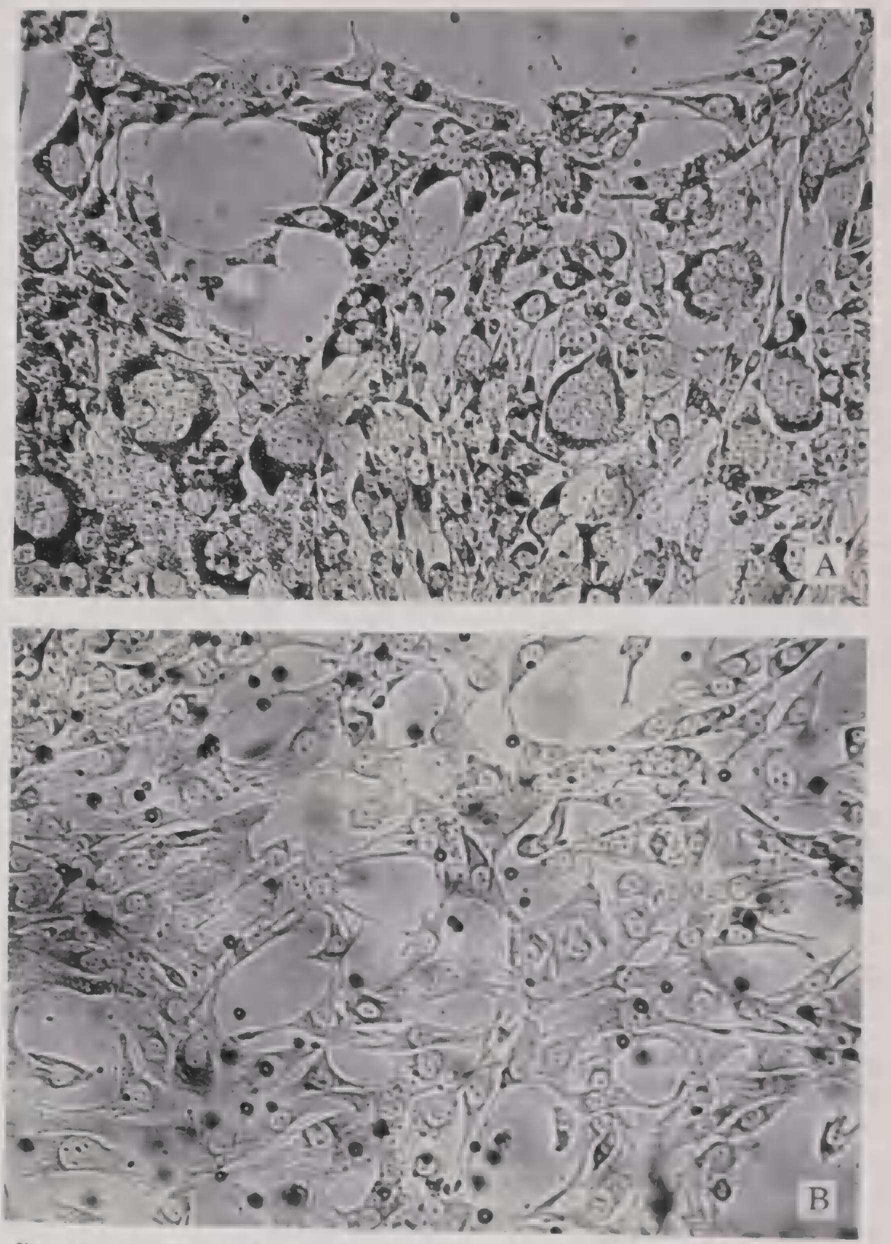

FIG. 13. *A*. Marginal area of culture of cells derived from mouse (Swiss) subcutaneous connective and adipose tissue and cultivated for ten months (eight passages) in 40 per cent Earle's salt mixture, 40 per cent horse serum and 20 per cent chick embryo extract. Note granular appearance of cytoplasm and presence of many multinucleated giant cells. × 127.

B. Marginal area of culture from another subline of same origin as the cells shown in *A*, after ten months (seven passages). × 127.

cancer, whereas the animals used by Earle have a high incidence of spontaneously occurring breast tumors. During the first sixty-eight days (two culture passages), the medium used on part of the cultures contained 0.002 per cent 20-methylcholanthrene; the remaining cultures were carried without carcinogen and served as controls.

The cultures treated with methylcholanthrene showed a marked inhibition from which they never recovered. In fact, they survived for only one culture passage (forty-five days) after the carcinogen had been removed. But cell strains derived from the original control cultures survived for as long as thirteen months (twelve passages), and by the fourth month they began to show evidence of cell changes remarkably similar to those described by Earle. Not only did the cells lose their long terminal processes, and become more amoeboid in appearance, but they also became increasingly coherent so that ribbons and epithelial-like membranes were formed. Besides, the rate of cell multiplication gradually increased, the lag period at the time of transfer gradually diminished, the cells became more granular in appearance, numerous multinucleate giant cells appeared (Fig. 13), and the life span of the cells became progressively shorter. But none of the cultures produced tumors when injected into mice of the same strain from which they had been derived. Either they were not truly malignant in character, or else the mice that were chosen for the experiments were not sufficiently homogeneous, genetically, to permit of positive takes in the absence of a definite tumor susceptibility. The fact that the transformations occurred in the untreated controls suggests either a trace contamination of these cultures with the carcinogen or the possibility that the malignant-like changes were in response to cell injury, which was most prevalent at all times. The cultures were extremely large, with dense central areas that soon became necrotic; the medium was completely heterologous and designed for a low rate of cell multiplication; and, though the cultures were fed regularly, during the early months they were subdivided and transplanted at intervals of not less than thirty days, a convenient but highly neglectful procedure that invariably resulted in a most unhealthy cell population. Eventually, after the transformations had occurred, the cells became much more active, lived for much shorter periods, and had to be transplanted much more frequently.

Haddow[123] has suggested that cancer cells arise and commence their career of proliferation as an adaptation to conditions that impair the growth of normal cells. Certainly the conditions existing in the culture experiments just described were such as to impair the growth of the original cells. And in this connection it is interesting to remember that Fischer[102] obtained carcinomata from the mammary glands of healthy mice when the glands were grafted, autoplastically and repeatedly, into different body regions. It is also interesting to note that Holtfreter,[139] who has made an extensive study of the biochemical nature of neural induction, feels that this phenomenon may be related to the mechanisms involved in the natural and parthenogenetic activation of the egg and in the induction of neoplastic growth. Holtfreter is of the opinion that the external stimuli capable of inducing all these phenomena are nonspecific but that all cause a temporary sublethal state of cytolysis that may determine the liberation and mutation of certain morphogenetic compounds (such as plasmagenes[63]) that are self-perpetuating.

One of the more interesting developments in the techniques of tissue culture in recent years has been the derivation, by Earle and his associates, of a pure strain of cells by single-cell isolation[233] and their demonstration of the ability of these cells to grow on perforated cellophane (see p. 188) in a fluid medium.[87] The isolations (Figs. 1 and 46) were made by freehand selection with fine capillary pipettes that were lined with a thin plasma coagulum. After the cells had been drawn into the pipettes, the capillaries were sealed, incubated for fifteen to twenty hours, then broken into segments that were embedded in a plasma coagulum consisting partly of a conditioned medium taken from large, rapidly-growing plasma cultures of the mother strain (see also p. 161). The cells were isolated from a strain of normal mouse mesenchyme cells that had been treated with methylcholanthrene and had subsequently become malignant.[77, 79] Eventually, and before the single cell strain had been isolated, the ability of the cells to produce tumors upon animal inoculation was lost.* But the cells retained their altered morphology and their ability to produce colonies from scattered, isolated cells when these were distributed over the surface of a layer of perforated cellophane spread on the floor of a Carrel culture flask. The experiments demonstrated that even the best medium used in tissue culture is inadequate for single, isolated cells, which require an environment that has been altered or added to by the presence of large cell populations. The experiments have also demonstrated that the inability of cultures to continue to grow indefinitely in a plasma coagulum must be attributed to the character of the coagulum[125] and not to the inability of the cells themselves to continue to multiply in a suitable environment. Unlike plasma cultures, which soon reach a maximum size, cells grown on perforated cellophane continue to multiply until they have covered the entire area of the cellophane. They may then be scraped or shaken into the medium and the resultant cell suspension may be diluted and used to seed new cultures.

Since viruses may be propagated in the presence of living cells, the tissue-culture techniques offer a direct means of studying the manner in which these agents infect cells, how they live and multiply within the cells, how they react to immune substances, and how they interfere with one another. As early as 1913 Steinhardt, Israeli and Lambert[244] demonstrated that vaccine virus retains its activity for more than a month in fragments of rabbit or guinea-pig cornea surviving in a drop of plasma. In 1925 Parker and Nye[204] inoculated fragments of rabbit testis with vaccine virus and cultivated them in plasma. In cultures of the eleventh generation these authors found 51,000 times as much virus as was present in the beginning. A year later Carrel[38] demonstrated that the virus of Rous chicken sarcoma was also capable of unlimited multiplication *in vitro.* By cultivating the virus with embryo pulp, fragments of spleen, and blood leucocytes in a plasma coagulum, he was able to keep on hand a constant supply of the active agent. In 1927 Carrel and Rivers[52] applied the same general procedure to the propagation of vaccinia virus. Infected rabbit testicle was ground in a mortar, added to chick embryo pulp, left for twenty-four hours in the refrigerator, and then inoculated into Carrel flasks containing a coagulum of adult fowl plasma. The cultures were washed every two or three days with Tyrode's solution and nourished with a dilute chick embryo extract. After periods varying from one to four weeks, the contents of the flasks were withdrawn, ground in a mortar, and titrated by the intradermal injection of rabbits. Culture flasks originally inoculated with from 25 to 250 intradermal units of virus per cubic centimeter were found, after eight days' cultivation, to contain from 10,000 to 100,000 units per cubic centimeter. These figures showed that the virus had undergone active multiplication. In the following year Maitland and Maitland[182] improved upon this method by the substitution of a fluid medium for the plasma coagulum.

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* Earle, W. R. Personal communication.

Since these observations were made, many different viruses have been propagated for longer or shorter periods in tissue culture, and in certain instances culture material has been used in the production of vaccines. To cite two examples: Rivers and Ward[226] produced Jennerian prophylaxis in man with a strain of vaccinia virus that was cultivated *in vitro* for several years without intercurrent animal passage. During this period, the virus lost its capacity to produce typical dermal lesions in the rabbit, though it still produced mild reactions in susceptible humans. And in 1932 Haagen and Theiler[119] reported the successful cultivation of a strain of yellow fever virus that had been greatly modified by intracerebral inoculation through many generations of mice. The medium consisted of chick embryo tissue and Tyrode's solution containing 10 per cent monkey serum. But attempts to cultivate unmodified strains of the virus led to negative results. Eventually, however, Lloyd, Theiler and Ricci[175] succeeded in establishing the unmodified Asibi strain in tissue cultures in which mouse embryo was used as the tissue component of the medium in place of chick embryo.

The cultures were incubated at 37° C. and subcultures were made every three or four days. After cultivation through eighteen subcultures in this medium, a separate subline was initiated in a medium containing minced whole chick embryo. After further cultivation through fifty-eight subcultures in the latter medium, the tissue component was modified to the extent that instead of using whole minced chick embryos, the brain and spinal cord were removed from the embryos before mincing. Somewhere between the 90th and 114th subcultures, however, a sudden change in the neurotropism of the virus occurred. The Asibi strain of yellow fever virus is one of the most virulent yet isolated, in both viscerotropic and neurotropic properties. But as a result of continued cultivation *in vitro*, it lost much of its pathogenicity[175, 247] and came to be used for human vaccination against yellow fever.[248] More recently, subcultures from this strain, designated 17D, have been used as seed material for the production, in developing chick embryos, of large quantities of living virus from which the human vaccine has been prepared.[241] Another instance of the use of tissue culture in altering the pathogenicity of a virus was provided more recently by the work of Koprowski and Lennette[148] who maintained the West Nile virus in cultures for eighty passages and obtained a marked attenuation of the ability of the virus to kill mice by extraneural inoculation. There was not, however, any obvious alteration in the lethal effect of the virus by the cerebral route.

Tissue-culture techniques have also been particularly useful in connection with the problem of virus specificity. It appears, for example, that the characteristic of species specificity possessed by many viruses is frequently retained and reflected by them when cultivated *in vitro*. Thus, Findlay[92] found that fowlpox virus, which is innocuous for mice and rats, does not multiply in cultures of their tissues. The same is true of fowl plague. Maitland and Maitland[183] also found that the virus of foot-and-mouth disease, which does not attack chickens, cannot be grown in cultures of minced chick embryos. In addition to their species specificity, however, some of the viruses also exhibit a pronounced cellular or tissue specificity. In 1924 Carrel made a study of the effect of filtered extracts of Rous chicken sarcoma

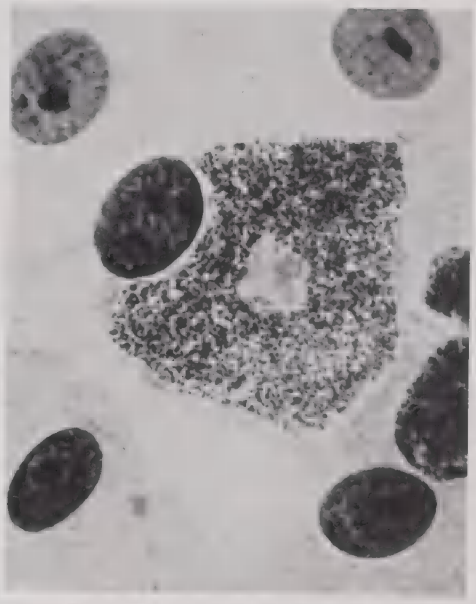

FIG. 14. Cell containing elementary bodies of psittacosis virus. (J. O. B. Bland and R. G. Canti.[21]) × 900.

on pure cultures of leucocytes and fibroblasts. When a culture of fibroblasts was inoculated with Rous virus, the cells did not usually undergo any change and the virus rapidly disappeared from the fluid in the flask.[37, 38] In leucocyte cultures, on the other hand, the virus multiplied with great rapidity.[36] Again, Hallauer[124] in 1931 found that the fowl plague virus multiplies in the presence of chick embryo skin, iris epithelium and brain, but not in pure cultures of fibroblasts or blood monocytes. In the same year, Hecke[134] reported that the virus of foot-and-mouth disease does not grow when fibroblasts alone are present; but the virus increases in amount when the culture medium contains minced embryo guinea-pig skin.

Tissue-culture studies have also contributed to our knowledge of the developmental morphology of virus inclusions. Thus, Bland and Canti[21] found that the elementary bodies of psittacosis virus (Fig. 14) pass through a definite cycle of development and that the plaque or inclusion body is essentially a colony of elementary bodies. Later, Bland and Robinow[22] reported a similar process of intracellular development when the elementary bodies of vaccinia were inoculated into cultures of epithelium from rabbit cornea. They found that the typical inclusions of vaccinia, Guarnieri bodies, are largely composed of elementary bodies. And Merling[191] has shown that virus particles phagocytized by leucocytes survive the death of the leucocytes and continue to grow. It is also interesting to note that in lymphogranuloma, vaccinia and psittacosis, the virus inclusions are cytoplasmic, whereas in Virus III and in herpes the inclusions are intranuclear.

Other investigations have been carried out in an attempt to determine the relative importance of humoral and cellular immunity in connection with the virus diseases. In 1929 Andrewes[1, 2] cultivated the Virus III infection of rabbits and found that the characteristic intranuclear inclusion bodies associated with this infection *in vivo* were also formed *in vitro*. Cultures were then prepared in which the virus was associated with (1) normal testicular cells and normal serum, (2) normal testicular cells and serum from an immune animal, (3) testicular cells from an immune animal and normal serum, and (4) testicular cells from an immune animal and immune serum. When the testicular cells from an immune rabbit were cultivated in immune serum, no inclusion bodies were formed. With normal testis and immune serum, the same result was obtained, provided the tissue was placed in the serum before the virus was added. If, however, the virus was allowed to remain in contact with the tissue for ten minutes, or longer, at 37° C. before the immune serum was added, typical inclusion bodies developed. These, however, were always fewer in number than in the control cultures with normal serum. When testicular tissue from an immune animal was combined with normal serum, inclusion bodies usually developed; when the tissue was well washed by a preliminary soaking in Tyrode's solution, inclusion bodies were always observed. Since similar results were obtained with herpes,[3] it appears that under the conditions of the experiments, and for the particular viruses employed, the humoral factor is decisive.

Meanwhile, however, Rivers, Haagen and Muckenfuss,[224, 225] working with the vaccinia virus, reported observations that were apparently divergent from those of Andrewes, although the differences might well have resulted from differences in technique. In the vaccinia work, normal rabbit corneas that had been infected with the virus, and were then cultivated in antivaccinal plasma, developed typical vaccinal lesions associated with Guarnieri bodies. In such cultures, it was found possible to demonstrate active vaccine virus after an incubation period of twenty-four or forty-eight hours. Furthermore, immune corneas inoculated *in vitro* with vaccine virus and then cultivated in normal or in antivaccinal plasma revealed either very mild lesions, or none at all. Sabin,[231] working also with vaccinia, later showed that both the virus and the antiserum are fixed by the tissue. If the fixation of the virus precedes that of the immune serum, infection of the cell is not prevented; if the fixation of the immune serum precedes that of the virus, infection is prevented.

The tissue-culture techniques have also been used to advantage in studying virus interference phenomena. Thus, Lennette and Koprowski[160] have shown that the 17DD High strain of yellow fever and the West Nile virus suppressed, more or less completely, the growth of influenza A virus in tissue culture, though interference in the reverse direction was not demonstrable. And Andrewes,[4] working with two biologically distinct strains of influenza A virus, found that whichever strain of influenza virus was added to the culture first could interfere with the growth of the second, added twenty-four hours later. But if the two strains were added simultaneously, the one present in the larger quantity suppressed the growth of the other.

In the study of antibody formation, the method of tissue culture has been employed by many investigators with widely divergent results. In 1912 Carrel and Ingebrigtsen[51] cultivated the bone marrow and lymph glands of guinea-pigs in homologous blood plasma to which were added small amounts of red cells from the goat. On the fifth day the culture fluid hemolyzed red cells from the goat without the addition of complement. After being heated at 56° C., the culture fluid lost its hemolytic power; but, by addition of complement, this lost power was regained. On the basis of these and other tests, it was concluded that immune hemolysins had been formed. Since that time, numerous investigators have employed this direct method of adding the antigen to the tissue at the time of explantation. Some have reported positive results, others negative. Salle and McOmie,[232] for example, recorded negative findings in experiments in which chick embryonic tissue and rabbit or guinea-pig spleen and lung were cultivated in media containing heterologous sera or foreign red cells.

Still other investigators have worked with tissues taken from animals that were injected with the antigen before explantation. Lüdke[180] injected killed cultures of typhoid bacilli into rabbits and guinea-pigs, removed fragments of the spleen and whole femur after from twenty-four to sixty hours and incubated them in various culture media. After from two to five days, he was able to detect agglutinins in extracts prepared from the spleen and bone marrow. Przygode,[221] Reiter[222] and Meyer and Loewenthal[192] have also reported antibody production in tissues removed after injection of the animal. In contrast, however, similar attempts by Kuczynski, Tenenbaum and Werthemann[150] were unsuccessful.

In 1937 further experiments of a similar nature were reported by the author.[210] When, for example, fragments of adult rabbit spleen were removed from animals injected two and three days previously with washed guinea-pig red cells, they were found to produce easily demonstrable agglutinins against guinea-pig red cells after four days' incubation in a fluid medium. For each culture (Fig. 58), approximately 100 mg. of tissue were suspended in 2 ml. of medium contained in large Carrel flasks. The medium consisted of equal parts of normal rabbit serum and Tyrode's solution that contained extra bicarbonate and four times the usual amount of glucose.

The serum in which the tissues were cultivated was usually taken from one or more untreated rabbits that had previously been found to contain at most mere traces of natural agglutinating antibodies against guinea-pig red cells. But occasional use was made also of autologous serum taken from the injected animal at the time the spleen was removed. As controls, all culture media were incubated without tissue for the period of the experiment. Additional controls consisted of duplicate cultures kept at 4° C., and also of cultures frozen three times at −49° C. before being placed for incubation at 37° C. Ordinarily all these yielded negative tests, whereas hemagglutinins produced by the tissues were detected in the culture fluid in various dilutions, sometimes as high as 1:320. Furthermore, no increase in antibody output was ever obtained by using serum from animals whose spleens had previously been found to produce antibodies in more than moderate amount.

Under the same conditions of cultivation, adult rabbit spleens taken from animals injected one to twenty-four hours previously with washed guinea-pig red cells failed to produce demonstrable antibodies. This result indicated the possibility that the production of antibodies outside of the body may be dependent upon certain reactions within the organism and that these reactions must reach a certain stage before the process can readily be completed *in vitro*.

Negative results were obtained also when spleens taken from animals injected twenty-four hours previously were cultivated in the sera of rabbits injected three days previously with a proportionate amount of the same antigen. The same results were obtained even when the serum in which the tissues were cultivated had been taken from animals whose spleens had produced demonstrable antibodies in dilutions as high as 1:256 and 1:320 when cultivated in normal serum. It appears, then, that if the serum of an animal injected three days previously contained certain necessary substances not present after one day, these substances were incapable of reacting upon tissues that had not undergone the same period of preparation.

Other experiments were made in which the spleen was perfused with the antigen by the direct injection of foreign red cells into the splenic artery. After from five to ten minutes the spleen was removed and cultivated in the usual manner. Again the results were negative.

Interesting observations were made also in the matter of animal individuality. When sera from different animals were used in the cultivation of tissues taken from a single organ, some proved to be excellent culture media (Fig. 58), whereas others were decidedly toxic. This factor, of course, affected the antibody output. It was found also that tissues removed from different animals that had been injected previously with proportionate amounts of the same antigen may, upon cultivation in a given sample of serum, remain in an equally good state of preservation and yet differ in antibody response.

The majority of the experiments described were also performed with bacterial antigens. The results were similar to those obtained when foreign red cells were used.

In view of the positive results obtained when the antigen was allowed to remain for from two to three days in the animal, the negative results obtained when this period was shortened have a very definite significance. They suggest that antibody production is a more complicated process than is usually assumed, and they also imply that it is not easy to demonstrate a production of antibodies *in vitro* unless the tissues have first been acted upon by some unknown mechanism within the body.

More recently, Landsteiner and Parker[154] used the procedures of tissue culture in the hope of obtaining new information concerning the origin of serum proteins, some of which are believed to be closely related to antibodies. To this end, connective tissue cells derived from the skeletal muscle of twelve-day-old chick embryos were cultivated for almost eight months (thirty-five weekly passages) in rabbit plasma and rabbit embryo tissue extract diluted with Tyrode's solution (Figs. 15 and 16). From time to time, fluids separated from these cultures were tested by means of precipitin reactions with immune sera obtained from rabbits after injections with chicken serum. These tests, which gave positive reactions that showed no tendency to diminish with an increasing number of culture generations, indicated that connective tissue cells can produce proteins that are identical with, or closely related to, serum proteins. The experiments also demonstrated that tissues cultivated in a foreign serum do not lose their species specificity.

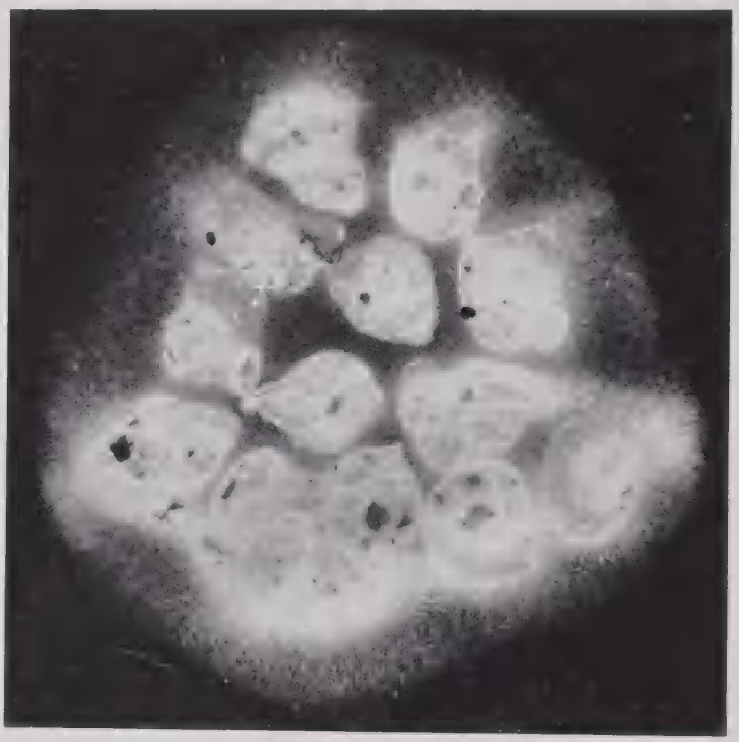

FIG. 15. Four-day culture of connective tissue cells derived from the skeletal muscle of a twelve-day-old chick embryo and cultivated for sixteen passages (weeks) in rabbit plasma. The denser areas represent fragments cut from a culture in the preceding passage; these fragments are completely surrounded by new outgrowth. (K. Landsteiner and R. C. Parker.[154]) ×7½.

In the present manual, an attempt will be made to outline briefly and concisely the essential details of the more basic techniques and practices that have been developed in many laboratories. In general, they include procedures for (1) the prevention of bacterial and chemical contaminations, (2) the preparation of media, (3) the maintenance of relatively constant physicochemical conditions in the medium, (4) the preparation of cultures for various special purposes, and (5) the simultaneous study of the morphology of cells, their physiological behavior, and the nature of the medium in which they live. But the tissue-culture techniques, like all others that serve a wide variety of special purposes, are undergoing constant change and revision. And this change will be even more rapid as they come to be used more extensively. It must be remembered, however, that tissue culture is not in itself a science or even a special

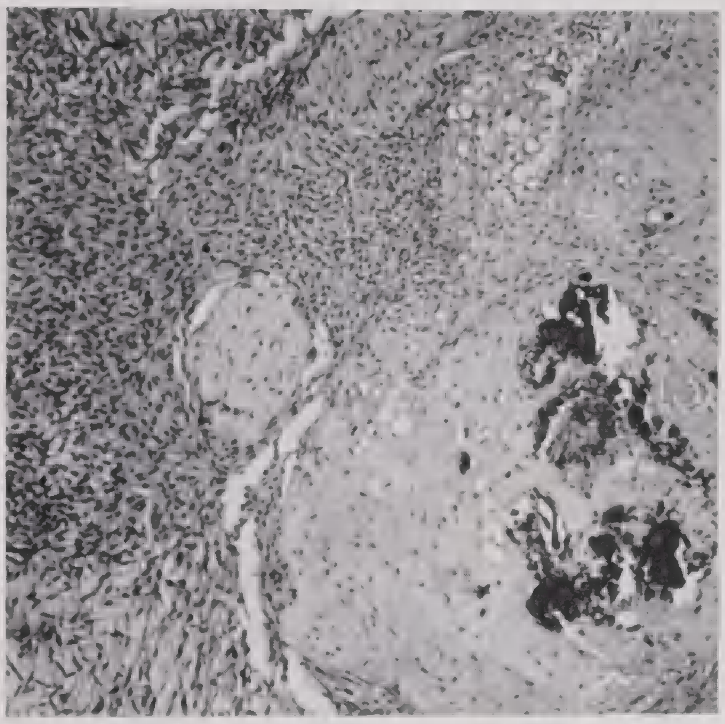

FIG. 16. Stained section of portion of a six-day culture of connective tissue cells derived from the skeletal muscle of a twelve-day-old chick embryo and cultivated for thirty passages (weeks) in rabbit plasma. (K. Landsteiner and R. C. Parker.[154]) ×115.

field of investigation. Instead, it is but one method of approach to innumerable problems in experimental biology and medicine. The effectiveness of the tissue culture techniques can be measured only by the uses to which they are put.

Chapter II

THE LABORATORY

THE first physiological laboratory in which surgical cleanliness could be maintained was probably that of Pavlov.* The principle was simple: It provided for a long series of connecting rooms, each room to serve its own particular purpose and, at the same time, to give additional protection against the penetration of dust into the last and most important, the operating room. The outermost room of the series was an antechamber for both the operating suite and a group of postoperative recovery rooms. The first room of the operating suite was used for washing and drying the animals; the next, for anaesthetizing the animals and preparing the site of operation; the third, for the sterilization of instruments and dressings, and for the preparation of the surgeons. The fourth and last was the operating room.

The main features of Pavlov's scheme were followed by Carrel in arranging his laboratories at The Rockefeller Institute for Medical Research, in New York. Carrel's laboratory contained two complete suites of connecting rooms in which two operating teams could work simultaneously. Like Pavlov's, each suite consisted of four rooms, but between the two suites, and serving both of them, were the general preparation and sterilizing rooms. In the first room of each series, the animals were anaesthetized and shaved; in the second, the site of operation was cleansed and the animals were draped for operation. The next room was for the preparation of the surgeon and his assistant, while the last was the operating room. After the operation, the animals were returned to the first room of the series where they were left to recover in special cages supplied with a continuous circulation of warm air. There were no windows in the side walls of these rooms; each was provided with skylights. The ceilings and walls of the operating suites were washable and painted dark grey in color, to absorb reflected light. The floors were black. All pipes, heating and ventilating appliances were built into the walls, to reduce the accumulation and dissemination of dust. Each operating room was furnished with a long, narrow operating table (15×50 ins.) and two or more auxiliary tables, all of proper height for seated workers. The operating rooms were also used as culture rooms.

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* Pavlov, I. P. The Work of the Digestive Glands. Trans. by W. H. Thompson. Ed. 2, 1910, London, Griffin.

For tissue culture, the foregoing plans may be simplified considerably without sacrificing the basic principle followed by Pavlov and Carrel, namely, the necessity of locating rooms for sterile work as far as possible from the contaminated air of the outer laboratory. In a laboratory where several workers are to be accommodated, it is advisable to have two or more culture rooms.

Each *culture room* should be not less than 7×10 feet in size and should be supplied with forced filtered ventilation, overhead lighting and a sliding door. In instances where the rooms are not supplied directly with filtered air, or where the air supply is inadequate, it will be necessary to draw air into the rooms by means of a fan placed near the ceiling. In any event, the inner end of the air duct should be fitted with a removable air filter (e.g., steel wool and oil) that may be changed at frequent intervals. The only outlet for escaping air should be around the sliding door; all other outlets should be sealed. Windows in the door and side walls are always an advantage for the laboratory staff and for casual visitors. The floors, walls and ceilings should be washable. The color of the walls should depend upon the nature of the lighting. Where adequate lighting can be arranged, the walls should be as dark as possible.

There are two main sources of bacteria in the air of culture rooms: (1) the organisms that are forcibly expired by the culture room personnel (see p. 69), and (2) dust scuffed from the floor or brushed from clothing. The rooms must be kept scrupulously clean with soap and water. Dry mops and brooms must not be used. Cleaning should be done at the end of the day so that there is ample time for dust to settle before the room is used again. Unpolished wood floors and those covered with linoleum can be treated with spindle oil to allay dust.[260] Spindle oil is a crude, heavy, petroleum oil used extensively in the cotton industry as a lubricant. One gallon of this oil is sufficient to cover 800 to 1000 square feet of floor space. The oil is noninflammable and odorless, and the floors are not slippery when dry. Applications should be made at intervals of two to five weeks after the initial oiling, thereafter at intervals of seven weeks. Oil is deleterious to rubber tile floors and is ineffective on tile or concrete.

From time to time the culture rooms should be tested for their bacterial content by exposing Petri dishes of nutrient agar for thirty minutes. If only four or five colonies appear in these dishes after forty-eight hours' incubation, the rooms may be considered fit for use. If the count is higher than this, special attention should be given to the air filter and the floors. Germicidal lamps, which have been used to considerable advantage in hospital operating rooms and elsewhere, should not be necessary for tissue culture work, which permits of more direct methods of protection.

Each culture room should be furnished with a long, narrow operating table (e.g., 18×60 ins.) and one or two auxiliary tables, all of proper height for seated workers and so arranged that two people may work facing each other (Figs. 36 and 43). For general purposes, stools have been found to be more satisfactory than chairs.

A *general preparation room* should be provided in close proximity to the culture rooms. The preparation room should be equipped with facilities for washing, drying and packing the glassware, instruments and other materials. There should be enclosed cabinet and drawer space for the storage of sterile materials and reserve supplies. This is a feature of particular importance. Even though materials are wrapped in paper and cloth prior to sterilization, they must be well protected until used; otherwise any dust that may accumulate on the outer coverings will be scattered in the culture room when the packages are opened.

A *sterilizing room* should be provided to house the hot-air and steam sterilizers. Owing to the heat usually generated by sterilizers, this room is likely to be unfit for more general purposes.

In addition to the facilities already mentioned, a well-equipped *chemical laboratory* should be available. There should be *dark-room facilities* for projection and photographic equipment. A *general laboratory* is required for the accommodation of the various incubators (unless a constant-temperature room is provided), for the study of cultures and for the keeping of records. There should also be *facilities for histological work.*

CHAPTER III

SPECIAL EQUIPMENT

MUCH of the equipment listed in the present chapter is in common use in biological laboratories or is easily obtainable from laboratory supply houses. A few of the items require the services of an experienced glass-blower; others, not otherwise available, may be manufactured by local craftsmen. Several items of special equipment are constructed on a principle that should be thoroughly understood if the techniques are to be used successfully. This principle, which involves safeguarding sterility by the use of obliquely placed openings, is illustrated in the design of the metal rack devised by Carrel for holding pipettes (Fig. 24). A sterile pipette, to be used more than once, is inserted in a glass tube for protection from the air. If the tube is placed flat on the table, the angle of approach is difficult and makes for loss of time. If the tube is supported in a vertical position, the pipette may become contaminated by dust particles falling from the air. But if the tubes are supported in a rack, at an oblique angle, the pipettes are protected adequately and may be handled rapidly and in quick succession with the least degree of effort.

This same principle was also followed by Carrel[35] in designing various types of culture flasks (Fig. 19) with oblique necks. Six or eight of these flasks may be opened simultaneously without fear of contamination from falling dust particles. While the flasks are open, it is only necessary to flame the necks from time to time to kill any organisms that may come to rest on the rims.

The same necessity for safeguarding sterility applies also to the handling of open medium tubes. When several ingredients are to be placed in a number of tubes, in different combinations, it is always convenient to introduce each ingredient into all the tubes before adding the next. Hence, they are supported in wooden blocks, in holes drilled at an angle of 45° (Fig. 17), and are flamed occasionally while open to the air.

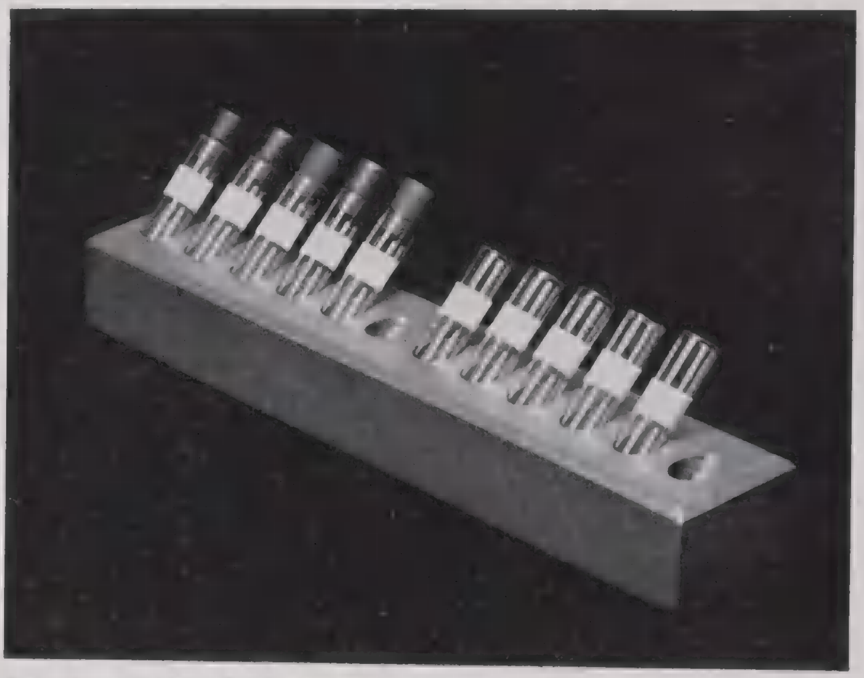

Fig. 17. Wooden block for the accommodation of tubes being filled with sterile media.

The following summary includes most of the items of special equipment that have been found useful for culture work:

Blocks, wooden, drilled with holes at an angle of 45°, for supporting glass tubes while they are being filled with sterile media. A block 16 ins. long, 3 ins. wide and 2 ins. thick can be drilled to accommodate a row of twelve tubes, each 18 mm. in diameter (Fig. 17).

Blocks, wooden, drilled with vertical holes for storing and transporting media contained in glass tubes. These may be about 1 ft. long, 2 ins. thick, and wide enough to accommodate two rows of tubes (Fig. 18).

Board, movable, ¾ in. thick, 8 ins. wide, and long enough to extend the full width of the table. This board, placed in a central position, serves as a raised platform (Figs. 37 and 43) to facilitate the handling of cultures.

Cellophane, perforated, upon which to cultivate tissues in flasks without plasma or fibrinogen clots (see p. 188).

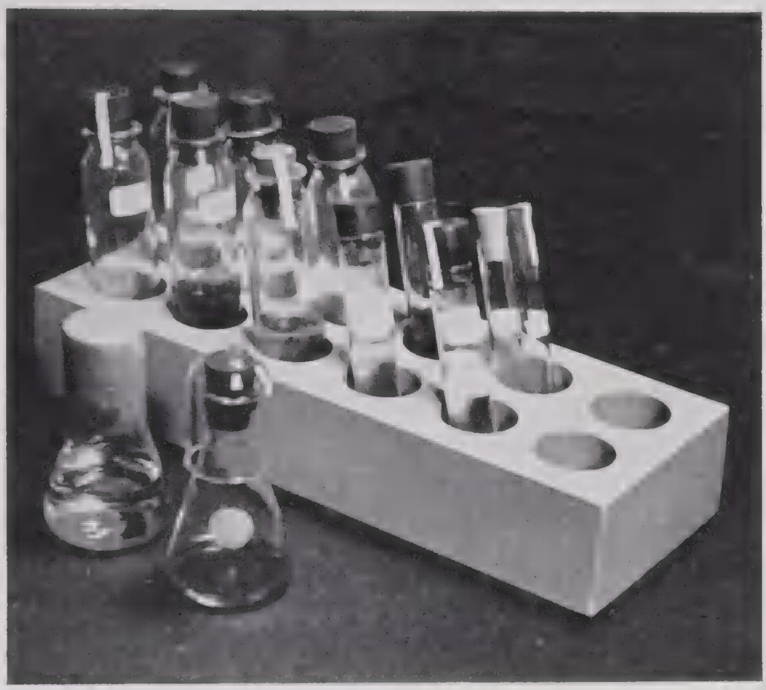

FIG. 18. Wooden block for storing and transporting culture media in tubes: also, 50-ml. Erlenmeyer flasks with their stoppers protected from dust by glass and aluminum caps.

Cloth goods: operating gowns, hand towels, table covers, animal sheets, and sterilizing covers in which to wrap materials sterilized by steam. The table covers and the larger animal sheets should be of black sateen. Dark coverings give a subdued effect that is beneficial for the eyes and essential for good culture work. Cultures are partially transparent and do not show up well against a light background. Dark coverings are also economical in that they do not require frequent laundering. Sterilizing covers are made in various sizes from a double thickness of unbleached muslin.

Coverglasses, micro, 24×24 mm., or 24×50 mm. (0.13 to 0.17 mm. thick), of noncorrosive glass, for small hanging-drop cultures to be observed, either living or fixed, under the higher magnifications of the microscope; 45×45 mm., of various thicknesses, for use with perforated slides and large depression slides; round covers, 22 mm. in diameter, for double-coverslip preparations.

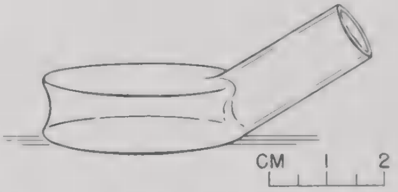

FIG. 19. Carrel D-3.5 culture flask.

Coverslips, mica, 24×50 mm., for hanging-drop cultures not intended for study at extremely high magnifications; 41×41 mm., for use with large depression slides, particularly double-coverslip preparations. Mica covers are nonbreakable and safer than glass. They may be washed, resterilized and used many times before they become too badly mutilated for further use.

Culture flasks, Carrel, Pyrex: Type D-3.5 is 35 mm. in diameter, 11 mm. high, with an oblique neck 10 mm. in diameter (Fig. 19); Type D-5 is 50 mm. in diameter, 12 mm. high, with an oblique neck 10 mm. in diameter (Fig. 42); Type "micro" is 35 mm. in diameter, 10 mm. high, with a straight neck 7 mm. in diameter (Fig. 20). At the union of the neck with the chamber of the micro flask, there are slight constrictions that prevent the culture fluid from running into

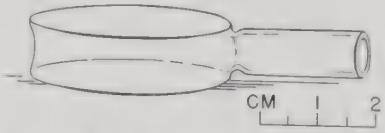

FIG. 20. Carrel "micro" culture flask.

the neck. The extreme thinness of the top and bottom renders it possible to study the cells with oil immersion lenses.

Culture slides, micro, 76×25 mm., with spherical concavity 22 mm. in diameter and 2 mm. deep, for hanging-drop preparations; 76×25 mm., with oval concavity 44 mm. long, 14 mm. at widest portion, and 2 mm. deep, for washing fresh tissues and cultures; 75×45 mm., with spherical concavity 36 mm. in diameter and 5 mm. deep, for double-coverslip preparations and cultures to be transferred to large perforated slides.

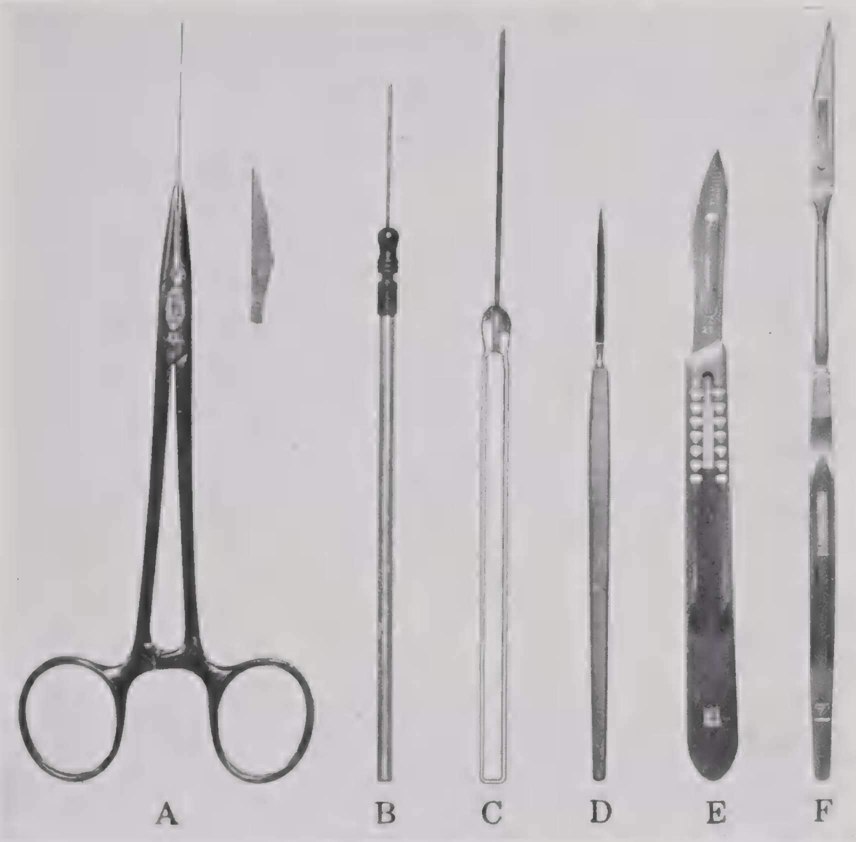

FIG. 21. Instruments used in tissue culture: (*A*) full-length fragment of double-edged razor blade held in surgical needle holder, as fair substitute for cataract knife; (*B*) platinum needle in screw-clamp, aluminum holder; (*C*) heavy spatula of tungsten steel fused into glass tubing; (*D*) cataract knife; (*E*) Bard-Parker holder No. 4 fitted with a No. 23 blade; and (*F*) Bard-Parker holder No. 7 fitted with a No. 11 blade.

Equipment for cleansing and sterilizing materials (see Chap. IV).

Equipment for the preparation of diluting fluids (see Chap. V).

Equipment for the preparation of plasma and serum (see Chap. VI).

Equipment for the preparation of tissue extracts (see Chap. VII).

Equipment for gassing the cultures with various mixtures of O_2, CO_2 *and* N_2 (see p. 153).

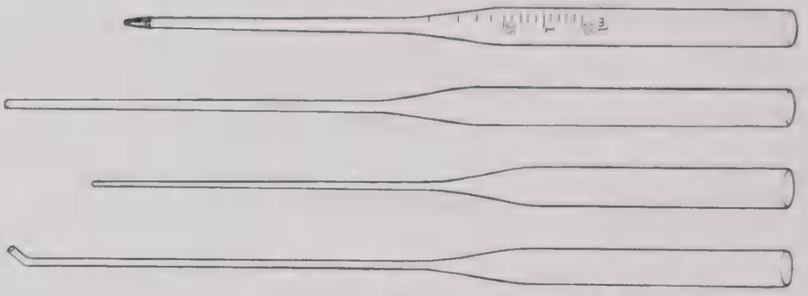

FIG. 22. Culture pipettes: 1.5-ml. pipette, graduated in 0.1 ml. intervals to extreme tip; coarse, ungraduated pipette; fine, ungraduated pipette; and bent pipette used to place tissue fragments in roller tubes.

Equipment for roller-tube cultures (see Chap. XII).

Gas burner, micro, of a type easily handled in flaming apparatus placed on any part of the table. The flame should be small in diameter and vigorous in its burning.

Gauze sponges, about 3×4 ins., made from 8-in. squares of surgical gauze so folded that there are no loose threads.[260]

Hot-plate, electric, for keeping paraffin wax in a liquid state over extended periods of time.

Incubators, the number and size depending upon individual requirements. When the incubator is large enough, or when a constant temperature room is available, the eggs for chick embryo extract and the cultures may be incubated together. Roller-tube cultures require a rotating device installed in a special incubator (Fig. 56) or in a constant-temperature room.

Instruments for culture work: cataract knives (Fig. 21*D*); fine and medium-fine forceps, both straight and curved; fine, sharp-pointed scissors; platinum needles made by fitting short lengths of platinum wire into screw-clamp holders

(Fig. 21*B*); needles or spatulas of tungsten steel (Fig. 21*C*); and replaceable blade scalpels, for use in preparing explants from fresh tissues (Fig. 21*E* and 21*F*). Full-length fragments of double-edged razor blades, held securely in surgical needle holders (Fig. 21*A*) provide fair substitutes for cataract knives.

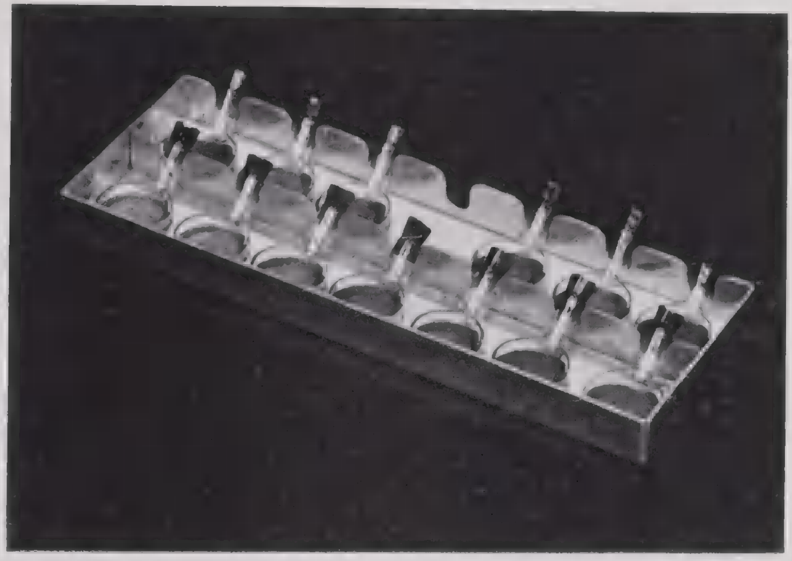

FIG. 23. Metal rack for the accommodation of D-3.5 culture flasks.

Masks, surgical (see p. 69).

Paraffin wax, for plasma tubes and sealing mixtures.

Pipettes: 1.5 ml., graduated at 0.1-ml. intervals to extreme tip; ungraduated, of approximately the same capacity, with coarse tips; ungraduated, of the same capacity, with fine capillary tips; ungraduated with bent tips, for roller-tube work (Fig. 22). The graduated pipettes are made from Pyrex glass by an experienced glass-blower; the others are drawn in the laboratory as required. For the capillary pipettes, "noncorrosive"* glass tubing is used, e.g., vial weight, specially selected, 8 to 8.5 mm. outside diameter. For the coarse, ungraduated pipettes, a heavier tubing is used, e.g., "noncorrosive," 1 to 1.3 mm. wall, 8.5 to 9 mm. outside diameter. The pipettes are packed for sterilization in large test tubes 50 mm. in

* Kimble Glass Co.

diameter and 250 mm. in length. The graduated pipettes may be *calibrated* in the laboratory by introducing successive 0.1-ml. amounts of mercury into the pipette, which is supported in an upright position over a thin layer of plasticine. As each 0.1 ml. of mercury is introduced, a pencil mark is made on a narrow strip of Scotch paper tape that is attached to the side of the pipette. The mercury is introduced by means of a 2-in. section of the tip of a 1-ml. serological pipette that is plugged tightly with cotton at the proper level to deliver 0.1 ml. and fitted with a small rubber bulb. A fine wire is used to dislodge air bubbles. After the mercury has been emptied out and the fifteen markings have been checked for accuracy (with 3.0 ml. of water, to magnify by thirty times any error that may be inherent in the short mercury pipette), the graduations are etched into the glass with a

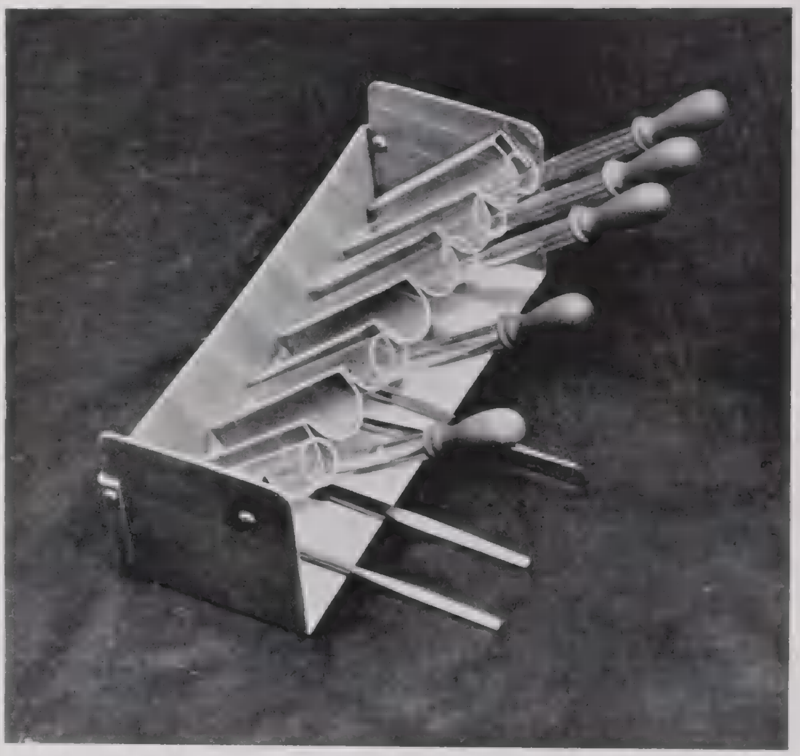

FIG. 24. Metal rack for the accommodation of sterile pipettes and instruments.

dental machine fitted with a carborundum disc. The pipettes are then washed and annealed at 560° C. for fifteen minutes to prevent subsequent breakage.

Plates, glass, about 3¼×4 ins., cut from ordinary sheet glass. These serve as sterile surfaces (Fig. 45*C*) for use in dissecting fresh tissues and in subdividing cultures removed from flasks. Eight or ten are packed for sterilization between single sheets of wrapping paper in a large Petri dish.

Rack, metal, for D-3.5 flasks (Fig. 23). When the flasks are fitted with rubber stoppers, they are often top-heavy and require support.

Rack, metal, for pipettes and instruments (Fig. 24). The top part of the rack forms a 45-degree angle with the lower part and is made with a series of deep grooves to accommodate glass tubes for holding pipettes. This arrangement protects both the pipettes and the compartment beneath from falling dust particles. The lower section may be used in emergency for the accommodation and protection of sterile instruments used in culture work. It can be flamed at frequent intervals. The glass tubes holding the pipettes are changed whenever the pipettes are changed.

Rack, wooden, for the accommodation of double-coverslip and perforated-slide cultures in the incubator (Fig. 41).

Rubber bulbs, for the pipettes, of about 2-ml. capacity.

Rubber caps for closing, temporarily, flask cultures that may become too acid. They are made by removing the constricted portion of the rubber bulbs, already described. The bulbous ends that remain (Fig. 44*A*) are partly filled with nonabsorbent cotton and packed for sterilization, open end down, in Petri dishes.

Spatulas, platinum, for manipulating cultures grown in flasks. They are made of heavy platinum wire (gauge No. 19, Brown & Sharpe's U.S. Standard Wire Gauge) and fused into glass tubing. The tips are hammered flat (but not too thin) and polished to extreme smoothness (Fig. 25). One foot of wire weighs from 4 to 5 gm.

Tubes, Pyrex, thin-walled, without rim, 18×100 mm., for

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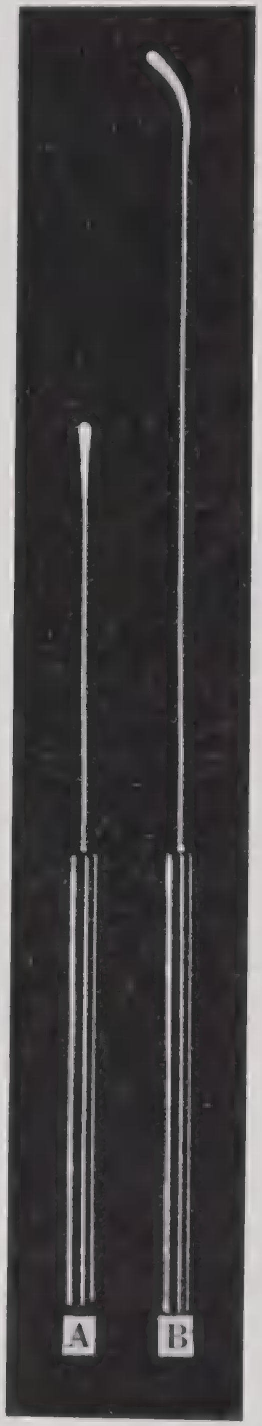

FIG. 25. Platinum spatulas: (*A*) type used for placing tissues in culture flasks; (*B*) type used in removing cultures from flasks at the time of transfer.

bleeding tubes, for media, and for use in the pipette racks; 25 × 100 mm., for the accommodation of pipettes and instruments lying flat on the table; centrifuge tubes, 50 ml., round bottom, with constricted necks, for large amounts of media. Corks and rubber stoppers to fit.

Vaseline (petroleum jelly), for hanging-drop cultures and sealing mixtures.

Chapter IV

CLEANSING AND STERILIZATION PROCEDURES

IN LABORATORIES where there are several assistants, it is advisable to have the washing and sterilizing done by the same individuals day after day. In any event it is good practice to place new, untrained assistants in the washing and sterilizing division of the laboratory and to have them remain there until they have thoroughly mastered that phase of the work. They may then be introduced to more specialized laboratory procedures and eventually to actual culture work, as assistants to experienced operators. Eventually those with special aptitudes for culture work will be able to work independently, and to train others. In this way the continuity of the laboratory program is assured. It is almost needless to state that a tissue culture laboratory comprised of a group of specialists, each trained in only one aspect of the work, is an inefficient laboratory that is in an almost constant state of crisis. Each member of the laboratory staff should be able, in an emergency, to perform any or all of the routine operations required for the care of the cultures. It is for this reason that new assistants should first be trained in the preparation room, the successful operation of which is so vital to the program of the entire laboratory.

If technical assistance is not available the investigator must arrange his work with the greatest care in order to make the most of his time. It is not impossible for one person working alone to carry out very successful experiments. Although progress may be slow, he will weigh the merits of any proposed experiment more thoroughly under these conditions. In laboratories where experiments are too easily made, they are often performed without due consideration for their actual value.

CLEANSING PROCEDURES

Tissue cells are extremely sensitive to toxic substances and, when cultivated *in vitro,* must be protected from every sort of contamination. The glassware must be chemically clean. The soap should be of a pure, white variety, and before being used, it should be reduced to a jelly. Two large cakes are cut into fragments and dissolved by boiling in water. The solution is diluted to a final volume of 16 to 20 quarts. It is then ready for use; on cooling, it will form a gel. Before the glassware is added, however, it should be redissolved in warm water. Some of the newer chemical detergents have also given satisfactory results.

Used glassware is rinsed under the tap to dislodge all solid matter, then boiled for ten minutes in water and soap jelly. After it has been rinsed in warm tap water, it is boiled again with soap, as before. It is now thoroughly rinsed with warm running water, placed for a time in distilled water, then in 95 per cent alcohol and set to drain in a drying oven. *New, unused glassware* is treated in the same manner except that it is first placed in dilute HCl (about 1 per cent) for several hours to remove any free alkali.

Glass coverslips, Carrel flasks and other glassware too fragile to be boiled, are rinsed with water, immersed in concentrated sulphuric acid containing small amounts (0.5 per cent) of sodium nitrate ($NaNO_3$) and sodium chlorate ($NaClO_3$), and heated to 90° C., after which the cleansing action is allowed to continue overnight at room temperature.[197] The following morning all of the articles except the flasks are again rinsed with many changes of tap water, then with distilled water and, finally, with 95 per cent alcohol. The flasks are treated with the alcohol *before* the final rinsing with distilled water. The coverslips are dried with a towel; the other glassware is allowed to drain in the drying oven.

Sintered (fritted) glass filters are also cleansed by immersing them in an acid cleaning solution composed of concentrated H_2SO_4 containing small amounts (0.5 per cent) of $NaNO_3$ and

$NaClO_3$.[197] The temperature of the cleaning solution is raised to 90° C., and the filters are left in the acid overnight at room temperature. They are then rinsed with distilled water, and 500 ml. or more of distilled water is drawn through them to remove all traces of acid. To make certain that the acid is completely removed, a *p*H determination is made on the last portion of the wash water.

New Berkefeld, Mandler and Selas filters are first soaked for a few hours in tap water, then brushed very gently with a soft brush (Selas filters, with a stiff brush) and tested for imperfections. Compressed air, at very low pressure, is passed through them under water; and the size and disposition of the air bubbles are noted. An even distribution denotes a perfect filter; occasional large bubbles indicate trouble. Before being sterilized, new filters are fitted to a suction flask (the filter candle is placed *inside* the flask by means of a single-holed rubber stopper placed on the stem) and flushed with 2 liters of distilled water drawn in through a rubber tube attached to a glass funnel. They are then submerged in 2 liters of distilled water, and the direction of flow is reversed. The waste water is caught in a water trap to prevent its flowing into the vacuum line.

Used Berkefeld and Mandler filters are flushed in both directions with distilled water, boiled for fifteen minutes in clean distilled water, flushed again in both directions with distilled water, tested with air pressure for imperfections, and reassembled for sterilization. If protein matter (serum, for example) has been passed through a filter, the last traces may be dissolved by treatment with a 1 per cent solution of pancreatin. From 100 to 200 ml. of pancreatin is allowed to flow through the filter by gravity. This will take several hours, perhaps overnight. The filter is then boiled for thirty minutes in sodium bicarbonate solution (approximately 1 tablespoonful to each quart of water), washed for at least ten minutes in running warm water, then flushed in both directions with distilled water and tested for imperfections.

Used Selas filters are cleaned with acid in the manner described for sintered glass filters. Selas filters can also be cleaned by ignition in an ordinary laboratory furnace at 1200° F. They should be thoroughly dried before they are heated, and should be allowed to cool in the furnace to room temperature before being removed. After they have cooled, they should be flushed well with distilled water.

Used Berkefeld, Mandler, Selas and sintered glass filters contaminated with infectious material are placed in clean distilled water and boiled for fifteen minutes. They are then cleansed in the usual way.

Used Seitz filters contaminated with infectious material are placed in a large vessel of water and boiled for fifteen minutes. The filter is then taken apart, the asbestos disc is discarded, and the metal parts are washed and reassembled with a new disc.

Used serological pipettes are rinsed under the tap and placed in acid cleaning fluid overnight. They are then washed for a day in an automatic pipette washer,[250] left overnight in distilled water, and allowed to drain in the drying oven.

Instruments are washed with soap and warm water, thoroughly rinsed with running water, placed in 95 per cent alcohol, and dried in an oven. Instruments are cleansed immediately after use, to prevent any organic material from drying on their surfaces. If it is necessary to scour them, it should be done with the finest nonabrasive material. Used knives are sharpened with a good razor strop. From time to time, it will be necessary to send them to an instrument maker for reconditioning. The use of whetstones should be avoided by any untrained person.

Mica coverslips are treated according to the procedures described for glassware, and dried with a linen towel. The towels are laundered with the same sort of pure soap used for the glassware since it is important to have them absolutely free from alkali. Mica is never placed in cleaning fluid.

New unused rubber stoppers are boiled in dilute sodium hydroxide (about N/2), rinsed in tap water, then boiled in dilute hydrochloric acid (about N/2), and finally rinsed very thoroughly in tap water. This treatment removes sulfur "bloom" and other surface impurities.

New rubber tubing that might come in contact with culture media is boiled in dilute sodium hydroxide (about N/2), rinsed in tap water, then boiled in dilute hydrochloric acid (about N/2). During the process of boiling, the hot fluids are circulated through the tubing with a syringe. This is done several times. After washing the outside of the tubing with hot tap water, the tubing is attached to a faucet and flushed with tap water for thirty minutes. A funnel is then attached to one end of the tubing and not less than 1 liter of distilled water is passed through it.

Used rubber tubing is washed thoroughly in warm tap water, then attached to a faucet and flushed with tap water for thirty minutes. Finally, a funnel is attached to one end of the tubing and not less than 1 liter of distilled water is run through it.

Sterilization of Materials

As a rule, sterilization is accomplished by heat (dry or moist), filtration, or chemicals. Dry heat is furnished by a gas or electric oven; moist heat, by an autoclave providing steam under pressure.

Although the principles of *sterilization by moist heat* are well known,[185, 252, 260] they are often disregarded in practice, sometimes with disastrous results. A frequent cause of trouble is the failure to realize that the sterilizing efficiency of steam under pressure depends entirely upon the moist heat that is present and not at all upon pressure. Steam that is saturated with moisture condenses on objects cooler than itself and, by giving up its latent heat, quickly raises them to its own temperature. When air is mixed with steam, this process is considerably less efficient because hot air sterilizes only by the slower action of heat absorption. The temperature developed in the sterilizing chamber of an autoclave is directly dependent, therefore, on the degree of air elimination from the chamber. When air is present, it results in the substitution of air under pressure for part of the steam under pressure, and heat penetration is especially poor at the bottom of the chamber where the air becomes concentrated.

For many years the performance of most steam sterilizers was measured by a pressure gauge and a thermometer mounted on top of the sterilizing chamber. This method, which is now obsolete, has been replaced by the use of a mercury thermometer placed in the discharge line. In nearly all modern sterilizers the steam enters the chamber at the back end, floats to the top of the chamber, compresses air in the bottom areas, and forces the air from the chamber through an opening at the front end. This system assures movement of steam from one end of the chamber to the other and from top to bottom. Air, being heavier than steam, tends to stratify beneath the steam and will move downwards, rapidly or slowly, depending on the density of the packing; and steam will follow as the air escapes. In order that the pressure shown on the gauge may actually represent the steam under pressure and not the air that has been compressed by the steam, the front drain valve of the autoclave must be left open one full turn for a period of five minutes to evacuate the air. Then the drain valve should be nearly, but not completely, closed and left so during the entire period of exposure. This procedure will permit a slight discharge from the chamber that will release the condensate and the pockets of air that gravitate from the load.

Certain modern steam sterilizers lack a hand-controlled discharge system but have thermostatic valves that are supposed to control automatically the discharge of air and condensate from the chamber. These valves are of the general type commonly known as steam traps. Unfortunately, however, they often become clogged. And serious mishaps can develop unless careful attention is given to a mercury thermometer that is located in the discharge line. Under the obsolete pressure system of control, exposure was usually timed as beginning when pressure had reached fifteen pounds. Under temperature control, the period of exposure should be timed as beginning when the thermometer in the discharge line indicates 115° C.

In a properly designed and operated steam pressure sterilizer, it is safe to assume that effective sterilization is achieved very shortly after the material reaches a temperature of 115° C. Thus, Hoyt, Chaney and Cavell[141] have shown that the spores of *Clostridium oedematiens* were destroyed (in direct contact with steam) as follows:

6 pounds saturated steam, 110° C., in 10 min. exposure
10 pounds saturated steam, 115° C., in 4 min. exposure
15 pounds saturated steam, 121° C., in 1 min. exposure

In practice, most articles sterilized by steam are exposed for thirty minutes at fifteen to seventeen pounds. But rubber gloves and solutions contained in small flasks should not be exposed for more than fifteen minutes.

TABLE I

TEMPERATURE OF SATURATED WATER VAPOR IN DEGREES CENTIGRADE AND DEGREES FAHRENHEIT CORRESPONDING APPROXIMATELY TO GAUGE PRESSURE IN POUNDS

Gauge pressure lbs. per sq. in.	*Temp.* °C	*Temp.* °F.
0	100	212
5	108	227
10	115	239
15	121	250
17	123	253
18	124	255
20	126	259

The proper way to cool down an autoclave after sterilization is to regulate the valve in the discharge line so that the pressure will be reduced to zero at a uniform rate in not less than six or seven minutes and not more than ten minutes. This procedure will give any fluids that may be present time to lose their heat at the same rate as the surrounding steam, without violent ebullition.

By placing *sterilization detectors* (for example, Diack controls) inside the actual packages, vessels or containers to be sterilized, and by making a series of trial runs with many different types of packing and loading, the operator will learn what temperatures should be indicated on the thermometer in order to achieve adequate sterilization of the entire load. Once the necessary temperature has been obtained, care must be taken to ensure a full period of sterilization. For greater efficiency, sterilizers should be equipped with automatic temperature-recording devices. In any event the time of sterilization should be noted in a special *record book* showing the sterilization number (the next in a series from 1 to 100, one number for each load), the date, and a few words indicating the general nature of the load. At the conclusion of the run, a metal tag should be placed on the door of each sterilizer. This practice will prevent other members of the laboratory from removing articles from a cold sterilizer without definite assurance that they have been sterilized. Also, the sterilization number should be written on each article *as it is removed* from the sterilizer.

The *Diack control* consists of a tablet of chemical material contained in a small hermetically sealed glass tube. Under adequate conditions of sterilization, the tablet in the glass tube will melt, fuse and change its shape. If, for example, the control is buried in a pack of goods in a location that is most inaccessible to contact with steam, and if it is found to be fused after sterilization, it is safe to assume that the entire load has been subjected to steam at a temperature and for a period of time necessary to bring about sterilization. Underwood[252] states that the Diack control will fuse in five to eight minutes at temperatures of 120° to 122° C. At 118° C., twenty to thirty minutes will be necessary. Where only one Diack control is used for each load, it should be placed in the center of the largest and most densely wrapped package in the load, and this package should rest on the tray in the bottom of the chamber midway between the front and rear ends. Because any air that remains in the sterilizer will gravitate to the bottom, this area is the coolest part of the sterilizer.

In *preparing the load for a steam sterilizer* the operator must make sure that adequate spaces are provided between packs to assure the passage of steam to the lower areas. It is best to place heavier packs on edge in the bottom tier and to make certain that they are not pressed tightly together.

If *sterilizing drums* are used the supplies should never be compressed into a tight mass, nor should the drum be loaded to full capacity so that pressure is required to close the cover. There should always be free space in the top of the drum when the cover is closed. Gowns, table coverings, towels and such should be placed in the drum lying flat so that when the drum is placed in the sterilizer on edge the load will also rest on edge to promote steam circulation. Drums should be lined with a double thickness of closely woven cloth (unbleached muslin). This covering of muslin surrounding the drum load serves as an air filter to eliminate dust as air is drawn in when the drum is removed from a warm sterilizer.

Dry heat is to be recommended only when direct contact of the materials with moist steam under pressure is impractical or when excessive heat can do no damage. Glassware is almost always sterilized by dry heat except when rubber fittings are present or when the items are unusually large (4, 10 and 20-liter bottles, which are autoclaved with a small quantity of water within them, to generate steam). Dry heat is also used for such materials as paraffin wax, oils, petroleum jelly and talcum powder, none of which can be sterilized properly in the autoclave because the moisture of steam cannot penetrate them.

Dry heat is most difficult to control; even in a well-designed and properly loaded oven, some materials may receive too much heat. Dry heat of 160° C. for sixty minutes is roughly equivalent to moist heat of 121° C. for ten to fifteen minutes. But because dry heat penetrates slowly and unevenly, exposures of at least ninety minutes (after the load has come to temperature) are preferred. Also, the heating elements should be so arranged that a whole hour is required to attain a temperature of 160° C. A shorter time may result in damage to glassware owing to uneven heating. Although prolonged exposures to 160° C. are not injurious to glassware, rapid changes in temperature are most destructive. For this reason, the oven doors should not be opened until the contents have cooled. And care must be taken to pack the oven in such a way that there is free space on all sides of the various items making up the load. Electric heat is preferred to that of gas, which is more difficult to control. Forced circulation of the air in a hot-air oven, by means of a blower, hastens heating and assures uniform conditions throughout.

The *wrapping and packing* of materials to be sterilized requires the utmost care. If materials are improperly wrapped and packed, sterilization may be incomplete or, if it is complete, they may become contaminated again by outside air even before they are removed from the sterilizer. As the materials cool, any air that is trapped within them, or within the coverings, will contract, and outside air will move in. For this reason, all containers and packages must be closed and covered in such a manner that the incoming air will be adequately filtered. All openings in glass apparatus must be plugged with cotton or fitted with corks. Whenever possible, corks are used instead of cotton to lessen the quantity of lint dispersed in the culture room when the tubes are opened. Finally, everything must be protected either with two layers of paper or with a double thickness of unbleached muslin.

Articles to be autoclaved are usually covered with muslin, though paper may be used if it is strong enough to withstand moisture. Articles to be sterilized by dry heat are covered with a grade of paper that can be heated to 170° C. without charring. Cellophane and other materials impervious to steam must never be used to cover articles to be autoclaved.

Care must also be taken to prepare articles to be autoclaved in such a way that no air will be trapped within them, for air that is trapped cannot be replaced by steam. Pans, jars and all other deep containers must be left at least partially open, wrapped in a double thickness of muslin and placed on their side in the sterilizer. Rubber stoppers are arranged between gauze pads in uncovered Petri dishes that are wrapped securely in a double thickness of muslin or paper and sterilized on edge. Rubber tubing is sterilized vertically, or arranged in a vertical spiral around rolled towels or an improvised cylinder, so that air can escape and steam can enter.

Glassware is sterilized by dry heat (in a gas or electric oven) for one and one-half hours at 160° C. Medium tubes and test tubes are fitted with corks and wrapped in paper, four or five to a package. Pipettes of the various sorts are plugged with cotton in the nipple-ends and placed in large test tubes 50 mm. in diameter. Strands of cotton extending beyond the ends of the pipettes are burned off before they are placed in the tubes. A ball of nonabsorbent cotton covered with gauze is placed in the bottom of each tube to protect the tip of the pipettes. The tops of the tubes are closed with gauze pads and wrapping paper, tied with string. Small culture slides (25 × 76 mm.) are sterilized, depression side down, over a layer of paper, in 6-in. Petri dishes. The covers of these and other dishes may be employed later to protect depression slides and glass plates that have been placed on the table for use. Large culture slides (45 × 76 mm.) are arranged, layer upon layer, with paper between them, in wire baskets that are packed, sterilized and stored while lying on one side, this arrangement to facilitate the eventual withdrawal of the slides, one by one as required. Glass plates are packed for sterilization (between sheets of paper of the same size) in 6-in. Petri dishes and wrapped in paper. Glass and mica coverslips are sterilized in smaller Petri dishes, wrapped in paper. Watch glasses are also sterilized in Petri dishes.

Erlenmeyer flasks are closed with corks that are covered with paper and tied with string. Where many flasks are used, protective pads can be made from one or more thicknesses of Fiberglas cloth hemmed with glass suture thread.* The cloth can be cut into squares with scissors, without fraying, if the pencil-marked lines of cutting are brushed lightly beforehand with glue or with cellulose acetate airplane dope (see footnote p. 86). When the Fiberglas pads become soiled, they can be cleaned in acid; and acid removes the glue.

Carrel flasks are plugged with nonabsorbent cotton and packed in groups of eight or ten in wire baskets that are wrapped with paper.

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* Porter, K. R. Personal communication.

Instruments are sterilized by dry heat for one and one-half hours at 160° C. The temperature should be as uniform as possible and the instruments should be kept well removed from the heating elements. Delicate instruments must never be overheated. If, under exceptional circumstances (in emergency only), instruments are sterilized by boiling, they are protected above and below with layers of gauze, covered with water and boiled for ten minutes. But they should be removed from the water while they are still hot, and dried in a sterile container, to prevent rusting.

Cataract knives, with their blade tips inserted in gauze pads, are sterilized, two or three together, beneath other gauze pads in flat metal boxes. Forceps and syringes are sterilized individually in glass tubes. Syringe needles are sterilized either in groups in Petri dishes, with their points buried in gauze, or individually in small test tubes with restrictions to hold the needle in place. The restrictions are made by flattening one side of the tube in a Bunsen flame.

Instruments for bleeding are assembled and packed for sterilization in a special container in the manner described on page 93. In laboratories where certain animal operations requiring a variety of instruments are performed at frequent intervals, it is well to have the instruments in a separate metal box, assembled layer upon layer, in the order of use, between gauze pads. If the boxes are large, their walls should be perforated. In any event, they should be wrapped in two thicknesses of paper.

Cloth goods (masks, gowns, table coverings, animal sheets, towels, etc.) and *rubber goods* (stoppers, tubing) are sterilized in an autoclave for thirty minutes at fifteen to seventeen pounds pressure (121° to 123° C). Rubber gloves (and nail brushes) are sterilized for fifteen minutes at fifteen pounds of pressure.

Solutions not altered appreciably by heating are autoclaved at fifteen to seventeen pounds pressure for thirty minutes or less, depending upon the size of the flasks. Flasks with a capacity of 1000 ml. or less should not be sterilized for longer than fifteen minutes; 50 to 250-ml. flasks should not be sterilized longer than ten minutes. During the actual period of exposure to steam, the solutions will not boil because of the pressure. Violent boiling and considerable loss of fluid will occur, however, if the pressure is reduced too rapidly. But if it is reduced too gradually the period of exposure may be too greatly prolonged. The flasks should never be filled more than two-thirds full and should be closed with loose-fitting cork stoppers that are covered with gauze pads and two layers of strong paper held in place with string. When the flasks are removed from the sterilizer, the corks should be inserted more firmly.

A more satisfactory means of sterilizing fluids by steam is provided by the Fenwal flask.* This type of flask has a heavy rubber cap with a central outlet that is fitted with a stainless steel secondary stopper. During sterilization the secondary stopper is inserted only part way, leaving an outlet for the release of pressure during the period of initial cooling. When the flask is removed from the sterilizer this secondary stopper is pushed all the way in, and the flask is sealed completely. As the flask cools to room temperature, a considerable vacuum is formed.

Solutions altered by heating are sterilized by filtration (see p. 67). These solutions include Tyrode's and other solutions of similar nature, doubtful samples of serum and solutions containing proteins and other heat-labile organic substances.

Berkefeld, Mandler, Selas, Seitz and sintered (fritted) glass filters are autoclaved for forty-five minutes at fifteen to seventeen pounds of pressure (121° to 123° C.).

Contaminated glassware is submerged in a vat of Phenolor† and autoclaved for thirty minutes at fifteen pounds of pressure (121° C.). It is then cleansed in the manner already described for used glassware.

Contaminated instruments are submerged, immediately after use, in a pan of Phenolor and boiled for ten minutes. They are then washed, rinsed and dried, to prevent rusting.

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* Macalaster Bicknell Co., Cambridge, Mass.

† Phenolor (E. R. Squibb & Sons) contains the following active ingredients: Orthophenylphenol, 13 per cent; soap, 13 per cent; and isopropyl alcohol, 3.8 per cent, by weight. The phenol coefficient is approximately 5.0. In addition to its high germicidal and antiseptic properties, it is noncorrosive, nonstaining and is free from any odor of phenol or cresol. But prolonged contact with the skin should be avoided. For general use, one volume of Phenolor is diluted with 32 volumes of water.

FILTRATION

Filters in general use for the sterilization of tissue culture media that cannot be heated are of four main types: (1) diatomaceous earth filters (Berkefeld, Mandler); (2) porcelain filters (Selas); (3) asbestos filters (Seitz); and (4) sintered glass filters (Pyrex).

Berkefeld filters are hollow candles of diatomaceous earth (Kieselguhr) cemented to a metal base. Because leaks often occur at the joint between candle and base, there is never complete assurance that a given filter can be relied upon to give a sterile filtrate. There are three grades: *V* (viel), a relatively coarse grade; *N* (normal), an intermediate grade; and *W* (wenig), the finest grade. The *V* filters are used for rapid clarification and for the removal of most of the bacteria in a liquid. The *N* filters usually remove all bacteria and allow the passage of most of the viruses into the filtrate. *W* filters retain bacteria and some of the larger viruses. Mandler filters, which are similar to the Berkefeld filters in appearance and function, are manufactured from Kieselguhr, asbestos and plaster of Paris.

Selas microporous porcelain filter candles are fine grained and smooth surfaced with the individual particles bonded together so that no loose material is present. Selas filters are available* in various shapes, sizes and grades of porosity (the 0.03 grade, type F.P. 54, ⅝×5 ins., is recommended for general use) and can be incorporated into many standard types of equipment, either as discs or as open-end cylinders. They can be cleaned by ignition or washed with a wide variety of acids or solvents. Sterilization of the filters can be accomplished by autoclaving, with the metal and rubber parts of the assembly attached and ready for use.

Seitz filters are made of asbestos and are obtained in pads and discs of different sizes and several grades of porosity. The

* Selas Corp. of America, Philadelphia, Pa.

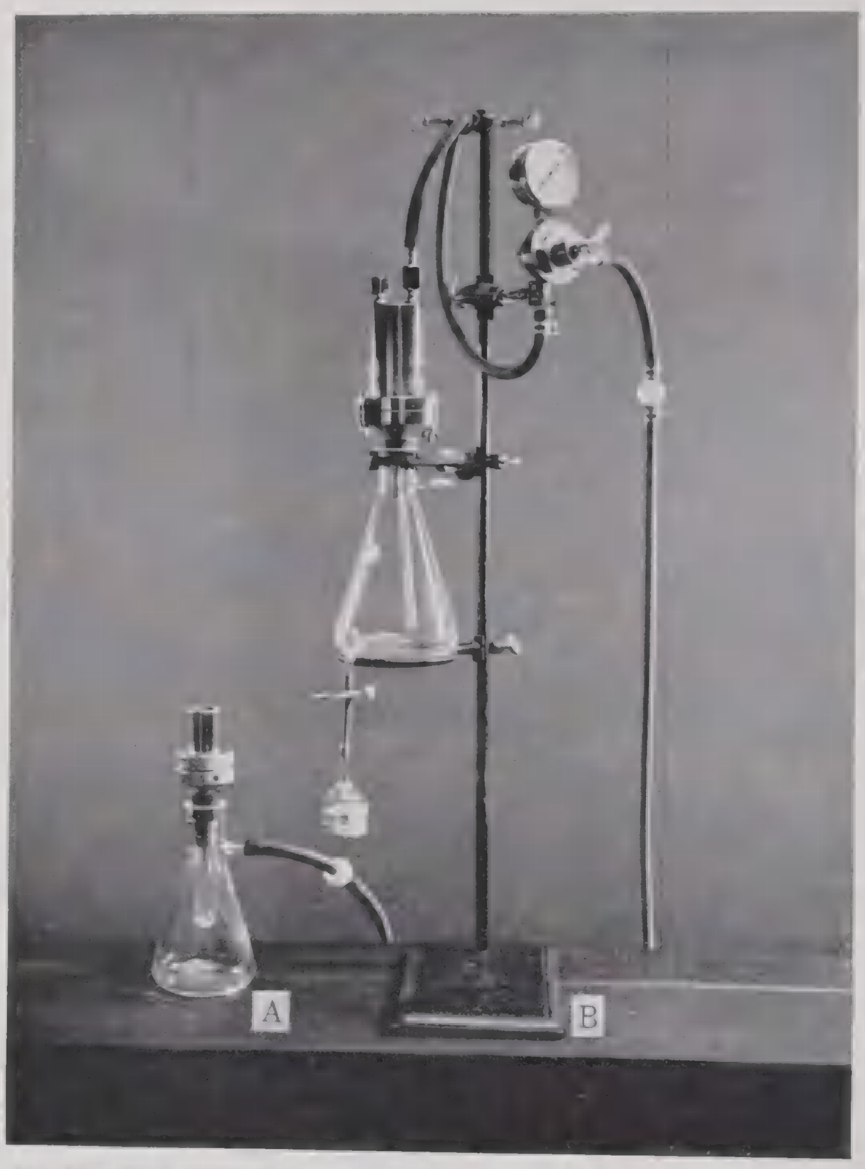

FIG. 26. Seitz method of filtration with especially prepared paper discs: (*A*) an apparatus arranged for the filtration of small amounts of material by suction (the inner tube is fitted to the filter outlet by means of a small stopper with a slit in its side); (*B*) an apparatus arranged for the filtration of large amounts of material by compressed air or nitrogen. (Care must be taken to use the minimum pressure required for the filtration of each type of fluid.)

E.K. (Entkeimung) or ST (sterilizing) grade is generally used for the filtration of materials in water solution. The discs are used but once, then discarded. They are held in a special metal container the joints of which fit tightly enough to prevent leakage. Because Seitz filters present no special cleaning problem, they are most convenient to use. It is important, however, to obtain sterilizing pads that are free from alkali.

Sintered (fritted) glass filters[197] are the nearest approach to the ideal filter in that they are easy to assemble and can be chemically cleaned before sterilization. They are made from glass that has been ground to very fine particles of uniform size, molded in the form of a disc, and heated to the sintering point without the addition of any other substance. The discs are then fused into glass vessels. The Pyrex brand of UF porosity is used for sterilizing purposes.

Suction provides the most convenient means of drawing liquids through filters. The air in a filter flask is withdrawn by aspiration through a water pump or an electrically operated vacuum pump. If a mercury manometer or a vacuum gauge is used, it is easily possible to observe and regulate the pressure during filtration. Filtration should be carried out as rapidly and at as low a pressure as possible. It is best not to use a greater pressure than that represented by 35 to 50 cm. of mercury. And regardless of the type of filter that is used, one should not filter too much material through one filter at a given time or continue the process too long. If the volume to be filtered is large or the rate of filtration is slow, it is better to use several filters instead of one. If bacteria are left in contact with a filter long enough, they will pass through. Before proceeding with filtration, it is advisable to remove coarse materials such as blood or tissue cells, precipitates, fibrin strands, and other large particles, by centrifugation.

USE OF FACE MASKS

Masking is the only effective means of controlling the discharges from the nasopharynx. An unmasked individual breathing through either the nose or the mouth expels only a few bacteria into the air. As soon as he talks or coughs, large numbers of organisms are expelled forcibly, chiefly in the form of droplets of saliva and bits of mucus. A mask catches the direct spray and accordingly reduces the number of bacteria in the air for the moment. As the droplets evaporate, the bacteria are deposited on the mask and are subsequently distributed in the room during quiet expiration. Thus the air expired through a mask that has been worn for a few minutes carries more bacteria than that from an unmasked individual who is breathing quietly.[132, 260]

The best mask is made of six layers of 42×42 gauze fashioned to fit the face so closely that the expired air is forced through the gauze rather than around it. The filtering efficiency of such masks increases markedly after repeated washing because of matting and felting of the fiber. One type of gauze mask is provided with a metal strip that can be fashioned to fit the nose to prevent fogging of glasses.

Masks should be changed frequently and boiled in soap and water before autoclaving. When they are dry, the masks are packed in a suitable glass container that is placed in the sterilizer on its side with a cloth or paper covering tied around the top. When the masks are eventually set out for use, the container is closed with its own glass cover that was sterilized in a separate wrapping.

HAND STERILIZATION

In preparing for animal operations, one scrubs the hands for at least five minutes with a hand brush and tincture of green soap under running warm water. The brush should be of good texture, with bristles neither too hard nor too soft; it is sterilized each time before using. The tincture of green soap is prepared as follows:

Soft soap	650 gm.
Oil of lavender	20 ml.
95 per cent alcohol, to make	1000 ml.

After the scrubbing has been completed, the soap is rinsed off thoroughly with running water and the hands are dried with a sterile towel. Next, they are soaked for a few seconds in either Dakin's or chlorazene solution and are dried again with a sterile towel. If gloves are to be used, the hands are powdered with sterile talc. Finally, and as an added precaution, the gloved hands are immersed in the antiseptic. If gloves are not used, a drop or two of 7 per cent tincture of iodine should be applied around the finger nails.

PREPARATION OF DAKIN'S HYPOCHLORITE SOLUTION

The antiseptic properties of sodium hypochlorite have been known for many decades. But the hypochlorite found in commerce not only has an extremely variable composition, but it contains free alkali and often free chlorine. Consequently, it is irritating when applied to living tissues. In 1915 Dakin* discovered a means of obtaining a solution free from caustic alkali and one in which the content of hypochlorite did not vary beyond 0.4 and 0.5 per cent. In preparing this solution, Dakin took advantage of the double decomposition of calcium hypochlorite and sodium carbonate. Later, even more uniform results were achieved by the electrolysis of a sodium chloride solution;† and Cullen and Austin‡ succeeded in making the solution from chlorine and sodium carbonate. Eventually, however, there appeared on the market various commercial concentrates of sodium hypochlorite prepared for use in homes and laundries and in the chlorination of water. It is easily possible[113] to prepare Dakin's solution from one or another of these concentrates.

From the standpoint of the present techniques, Dakin's solution, if properly prepared, has many advantages. It may be used

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*Dakin, H. D. *Brit. Med. J.*, 1915, 2, 318.

†Carrel, A., Dakin, H., Daufresne, M., Dehelly, G., and Dumas, J. *Presse méd.*, 1915, 23, 397.

Keen, W. W. The Treatment of War Wounds. Ed. 2, Philadelphia, W. B. Saunders Company, 1918.

‡Cullen, G. E., and Austin, J. H. *Proc. Soc. Exper. Biol. & Med.*, 1917-18, *15*, 41.

frequently without rendering the skin rough or uncomfortable. Because it breaks down very readily in contact with protein materials, it causes no lasting damage to normal body tissues but destroys immediately isolated, and hence more vulnerable, bacterial organisms. During the course of an operation it may be used freely on the most delicate tissues without killing the constituent cells. In short, whenever and wherever applied, it affords the utmost protection from contaminating organisms, with the least damage to the tissues.

Definition. Dakin's solution contains not less than 0.4 per cent and not more than 0.5 per cent sodium hypochlorite ($NaOCl$). If the percentage of sodium hypochlorite is less than 0.4 per cent, the antiseptic power of the solution is too low; if it is greater than 0.5 per cent, the solution is irritating. Dakin's solution is weakly alkaline (pH about 8.5 to 9.0). If the solution is alkaline to powdered phenolphthalein, it is irritating; if it is acid to an alcoholic solution of phenolphthalein, it is too unstable.

Preparation by Dilution of a Strong Hypochlorite Solution. Commercial concentrates of sodium hypochlorite are sold under various trade names (such as Clorox) and consist usually of 5 or 6 per cent solutions of $NaOCl$. One volume of the commercial solution is diluted with 9 volumes of water and sufficient $NaHCO_3$ is added to make a final concentration of 0.5 per cent. This procedure usually results in a solution of 0.5 per cent $NaOCl$ with a pH of 8.8 to 9.0.

In the author's laboratory Dakin's solution is prepared from a stronger concentrate containing approximately 12 per cent $NaOCl$. The procedure is as follows: 144 ml. of the concentrated solution are added to 1 to 2 liters of water; 4 ml. of 6N HCl are added to a small volume (400 to 500 ml.) of water; and 32 gm. of $NaHCO_3$ are added to another small volume of water. Finally, the three solutions are mixed and sufficient water is added to make 4 liters. The resulting Dakin's solution has a hypochlorite concentration of 0.4 per cent and a pH of 8.8.

Titration. With a bulb pipette, 10 ml. of the solution are measured into a beaker or Erlenmeyer flask containing from

50 to 100 ml. of tap water. Next, 5 ml. of a 10 per cent solution of potassium (or sodium) iodide and 5 ml. of glacial acetic acid are added. With vigorous agitation of the beaker or flask, the free iodine is titrated with N/10 sodium thiosulphate; and starch is used as an indicator. The amount of N/10 thiosulphate required to decolorize the solution, multiplied by the factor 0.0372, gives the percentage of sodium hypochlorite. For example:

$$10.77 \text{ ml.} \times 0.0372 = 0.4 \text{ per cent.}$$
$$13.43 \text{ ml.} \times 0.0372 = 0.5 \text{ per cent.}$$

If the solution is too strong, it may be diluted to 0.5 per cent sodium hypochlorite with 1 per cent sodium carbonate. But the designated amount of carbonate is planned to give, at a concentration of 0.5 per cent sodium hypochlorite, the minimum degree of alkalinity consistent with stability. If chlorine has been introduced in such excess that 3 or 4 ml. more than the desired amount of N/10 thiosulphate are required in titration, the solution should be rejected. As an alternative procedure, use may be made of the arsenious oxide titration method.*

Tests for Alkalinity. (1) A few crystals of powdered phenolphthalein are shaken vigorously with about 5 ml. of the solution to be tested. It should remain entirely colorless. If it becomes red in color, the solution is too alkaline and must either be discarded or the excess alkalinity neutralized.

(2) About 0.5 ml. of a 1 per cent alcoholic solution of phenolphthalein is squirted from a dropper into a test tube containing about 5 ml. of the solution to be tested. The solution should show a red color that will soon disappear. If there is not at least a momentary flash of red color, the solution has so low an alkalinity that its hypochlorite content will rapidly diminish. *Note: These tests are entirely independent, but equally important.*

If the solution is stored in darkness in the refrigerator, it may be kept for two or three weeks, with little change in its hypochlorite content. At room temperature, it decomposes very rapidly. When several different antiseptic solutions are in use in the same laboratory, Dakin's solution may be differentiated by coloring with 1 ml. of a 0.5 per cent solution of potassium permanganate per liter.

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* See Methods of Analysis, A.O.A.C., Ed. 5, Washington, 1940.

CHAPTER V

DILUTING FLUIDS, *p*H DETERMINATIONS AND OSMOTIC PRESSURE

CULTURE media may be classified roughly as being either nutritive or nonnutritive, and either natural or synthetic in origin. Most nutritive media are derived, directly or indirectly, from the organism. Some of these (such as blood serum, blood plasma, and the various body exudates) occur naturally, others are extracts of tissues and organs, while still others are prepared synthetically from chemically known constituents. Diluting fluids (such as Tyrode's solution, Earle's solution, Simms' solution) are nonnutritive and are used both as diluents for the various types of nutritive media and as irrigating solutions.

Before proceeding to more practical matters, it may be interesting to remember that the early salt mixtures were developed by physiologists as perfusion fluids for the study of heart function, muscle contraction and peristalsis. The English physiologist, Sydney Ringer,[223] in a series of classical papers published between 1880 and 1895, showed that an excised frog's heart would continue to function normally for a considerable period if perfused with a fluid containing sodium, potassium and calcium, these three cations being used in proportions similar to those existing in sea water and in the blood of higher animals. Since then, it has been found that these same three cations, acting separately or in combination with one another, help to regulate such vital phenomena as osmotic pressure, the permeability of cell membranes, the quickness of response to cell stimulation and the adhesiveness of cells that makes for tissue integrity.

Before Ringer began his investigations it had been known that the isolated heart of the frog would cease to beat if placed in 0.75 per cent sodium chloride. But Ringer found that if a small amount of calcium chloride was added to the solution, the heart beat recovered to a degree, though not for long. If, however, a little potassium chloride was added, the heart action remained normal for a much longer period. It was further found that although each cation was toxic when present alone, the toxic action of any one of them, even when present in excess, was neutralized by the presence of the correct physiological concentration of one of the others. These observations led Ringer to formulate the principle of a "balanced" salt solution, in which one cation is able to antagonize or neutralize the toxic effects of another. It is important to note, however, that ions that are antagonistic to one another in relation to one biological process may not be antagonistic in relation to another biological process.

In addition to the chlorides of sodium, potassium and calcium, Ringer's solution finally came to contain sodium bicarbonate, which was used to imitate the slight alkalinity present in blood. Ringer tried to substitute, for the chlorides, other salts of calcium and potassium, but found that, in the presence of sodium chloride, the other anions produced the same effects. The composition of Ringer's solution for the frog's heart is usually given as follows:

	Gm.
$NaCl$	6.50
KCl	0.14
$CaCl_2$	0.12
$NaHCO_3$	0.20
Water to make 1 *liter*	

In 1901 Locke[176] devised a modified Ringer's solution adequate for maintaining for a time the excised hearts of dogs, cats and similar laboratory animals. To correspond with the higher osmotic pressure of their bloods, the salts were used in slightly greater concentration. Also, a small percentage of glucose was added. The Ringer-Locke solution most commonly used has the following composition:

	Gm.
NaCl	9.00
KCl	0.42
$CaCl_2$	0.24
$NaHCO_3$	0.20
Glucose	1.00
Water to make 1 *liter*	

In 1910 Tyrode[251] devised a modified Ringer-Locke solution to which he added magnesium and monobasic sodium phosphate. Magnesium, he found, was effective in maintaining the contractions of mammalian intestine; and the sodium phosphate improved the buffering capacity of the solution. Over the years, many different modifications of Tyrode's solution have been devised.

TYRODE'S SOLUTION*

This solution is more generally used than any other diluting mixture. It is particularly useful as a diluting fluid for serum and other natural media, or as a vehicle for the organic constituents of synthetic media. It has the following composition:

	Gm.
Sodium chloride (NaCl)	8.00
Potassium chloride (KCl)	0.20
Calcium chloride ($CaCl_2$)	0.20
Magnesium chloride ($MgCl_2.6H_2O$)	0.10
Sodium phosphate monobasic ($NaH_2PO_4.H_2O$)	0.05
Sodium bicarbonate ($NaHCO_3$)	1.00
Glucose	1.00
Water (glass distilled) to make 1 *liter*	

Freezing point −0.62° C

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* In Tyrode's original publication[251], it is not stated whether the calcium chloride, the magnesium chloride and the sodium phosphate were anhydrous, though it is unlikely that they were. In any event, Tyrode did not indicate the number of molecules of water of crystallization that may have been present. While it is possible, therefore, that the amounts given here may not correspond exactly with the amounts actually used by him, the present formula is the one most commonly used.

Many investigators experience considerable difficulty in preparing this solution. The usual complaints include cloudiness and high alkalinity. If a few simple precautions are taken, however, both these difficulties may be avoided. Only reagent salts should be used. Approximately 850 ml. of glass distilled water are placed in a 1000-ml. graduate and the various ingredients are added to the water, one at a time, in the order designated. Each substance is dissolved completely and distributed throughout the entire volume of the solution before the next is added. This precaution is necessary in order to avoid high local concentrations of any one ingredient. When the mixture has been completed, water is added to make 1 liter of solution. The *p*H is then tested with a *p*H meter or in the manner described on page 83.

The *p*H of Tyrode's solution should range from 7.4 to 7.8. If the *p*H is higher, it is probably because the sodium bicarbonate contains some sodium carbonate. Sodium bicarbonate originally free from carbonate may form the latter upon standing, particularly if exposed to the air. This change does not, however, render the salt unfit for use. By the addition of dilute hydrochloric acid, the *p*H may be adjusted* after the solution has been made up. But this adjustment may be made before the solution is completed if the sodium bicarbonate is dissolved separately in about 100 ml. of water and carbon dioxide gas is bubbled through it. This process reconverts the carbonate into bicarbonate. The adjusted material is then added to the other ingredients.

Because Tyrode's solution contains bicarbonate and cannot be heated, it is sterilized by filtration. After filtration the solution is stored in the refrigerator in rubber-stoppered, pyrex tubes (50-ml. centrifuge tubes with constricted necks). The use of tubes is recommended so that only small samples of the solution need be unstoppered as required. Each time a sample is opened, the loss of a certain amount of carbon dioxide tends to increase the alkalinity. It is better, therefore, not to save an opened tube from one day to the next, which is also a reasonable precaution from the standpoint of sterility.

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* A solution made with Merck's sodium bicarbonate usually gives the proper pH without adjustment.

DOUBLE TYRODE'S SOLUTION

It is sometimes convenient to have Tyrode's solution of double strength. Such a solution can be mixed with an equal volume of the water solution of a substance to be used in the culture medium, and will still give an isotonic mixture. It is made in exactly the same manner as ordinary Tyrode's solution, except that twice the amount of each ingredient is added to the original volume of water.

TYRODE'S SOLUTION WITH EXTRA GLUCOSE

When large quantities of tissue are cultivated in a small volume of medium (see p. 204), it is usually desirable to increase the glucose content of the Tyrode's solution. If the amount of glucose is increased to 3 gm. (see formula for Tyrode's solution), the sodium chloride should be reduced to 7.78 gm. in order to give an isotonic solution. If 4 gm. of glucose are used, the sodium chloride should be reduced to 7.70 gm. In both instances, the other constituents are added in the same amount and in the same order as in normal Tyrode's solution. Both of these solutions have a freezing point ranging from $-0.61°$ to $-0.65°$ C.

GLUCOSOL SOLUTION

Glucosol has exactly the same composition as Tyrode's solution except that the sodium bicarbonate is omitted. It is slightly acid in reaction and has very little buffering action. It takes on the *p*H of the material with which it is mixed and can be sterilized by heating in boiling water or by autoclaving. While glucosol should not form a large part of the culture medium, it may be used when the rest of the medium is alkaline or contains an abundance of buffering materials. It is valuable as an irrigating mixture for tissues and cultures and as a solvent for materials unstable in alkaline media.

SIMMS' SOLUTION

This solution[240] was especially devised for tissue cultures, as a substitute for Tyrode's solution, and is characterized by a reduced concentration of calcium and an increased concentration of phosphate. It may be used as a diluting mixture for serum and other natural media or as a vehicle for the organic constituents of synthetic media. Because the solution contains bicarbonate, it must be sterilized by filtration. As when Tyrode's solution is used, the *p*H of the medium is controlled by means of gas mixtures containing an appropriate amount of carbon dioxide.

	Gm.
Sodium chloride ($NaCl$)	8.00
Potassium chloride (KCl)	0.20
Calcium chloride ($CaCl_2.2H_2O$)	0.147
Magnesium chloride ($MgCl_2.6H_2O$)	0.203
Sodium phosphate dibasic (Na_2HPO_4)	0.213
Sodium bicarbonate ($NaHCO_3$)	1.01
Water (glass distilled) to make 1 *liter*	

EARLE'S SOLUTION

This solution[77] contains more bicarbonate than Tyrode's solution and has therefore a higher buffering capacity. Because Earle used anhydrous salts, the amounts given by him for magnesium sulfate and sodium phosphate have been adjusted for the use of hydrated salts. The solution is sterilized by filtration.

	Gm.
Sodium chloride ($NaCl$)	6.80
Potassium chloride (KCl)	0.40
Calcium chloride ($CaCl_2$)	0.20
Magnesium sulfate ($MgSO_4.7H_2O$)	0.20
Sodium phosphate monobasic ($NaH_2PO_4.H_2O$)	0.14
Sodium bicarbonate ($NaHCO_3$)	2.20
Glucose	1.00
Water (glass distilled) to make 1 *liter*	

GEY'S SOLUTION

This solution* has a higher potassium and a lower calcium level than Tyrode's solution and is not highly buffered. The *p*H of the solution, or of the medium to which it is added, is controlled by means of gas mixtures containing an appropriate amount of carbon dioxide. It has the following composition:

	Gm.
Sodium chloride (NaCl)	8.00
Potassium chloride (KCl)	0.38
Calcium chloride ($CaCl_2$)	0.13
Magnesium chloride ($MgCl_2.6H_2O$)	0.21
Sodium bicarbonate ($NaHCO_3$)	0.25
Potassium phosphate monobasic (KH_2PO_4)	0.025
Sodium phosphate dibasic ($Na_2HPO_4.12H_2O$)	0.30
Glucose	1.00
Water (glass distilled) to make 1 *liter*	

FILTRATION OF DILUTING FLUIDS

A satisfactory arrangement for the filtration of Tyrode's solution, and other fluids to be filtered and tubed in large quantities, is shown in Fig. 27. It consists of a Seitz filter and a Pyrex suction flask with an extra outlet at the bottom. To this lower outlet is attached a small length of rubber tubing (eventually fitted with a pinch-clamp) and a bell-tube for use in filling the storage tubes.

The apparatus is assembled as follows for sterilization: To the stem of the filter is attached a single-holed rubber stopper that will fit the neck of the suction flask. The stopper and the filter outlet are protected with a gauze pad that is covered with paper and securely tied with string. The entire filter is then wrapped in two thicknesses of muslin. The suction flask assembly is sterilized as a separate package. The glass bell-tube is attached to the lower outlet of the suction flask by means of a short piece of rubber tubing. The bell itself is stuffed with gauze-covered nonabsorbent cotton, covered with paper and securely tied with string. A short section of pressure tubing and a cotton filter (double-end tube filled with nonabsorbent cotton) are attached to the side arm. Finally, the entire unit is wrapped in a double thickness of muslin. And both the filter and the matching suction flask are sterilized for thirty minutes at 121° to 123° C. (fifteen to seventeen pounds of pressure).

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* Bulletin of The Tissue Culture Association, March 16, 1949.

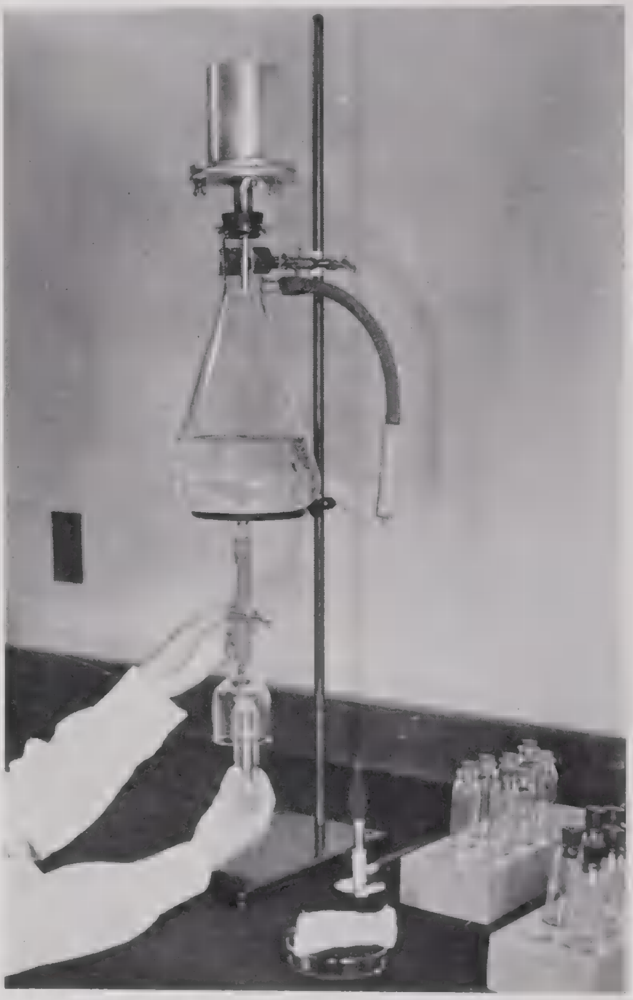

FIG. 27. Seitz filtration by suction, and the procedure followed in filling storage tubes.

The Pyrex storage tubes are fitted with corks and wrapped, in groups of four or five, for sterilization by dry heat (one hour at 160° C.). Rubber stoppers, for closing the filled tubes, are placed in an uncovered Petri dish (small ends against the bottom). The dish is then covered with a gauze pad and wrapped in a double thickness of unbleached muslin for sterilization in the autoclave.

When the entire quantity of Tyrode's solution has been filtered into the flask, the suction is turned off and about ten minutes are allowed for the equalization of the outside and inside pressures before the suction tubing is disconnected. Then, by means of the bell-tube, the solution is drawn off into the sterile storage tubes. This simple device eliminates the great danger involved in pouring fluids from the neck. The bell-tube is designed to protect the open mouth of the storage tube from falling dust particles while the tube is being filled (Fig. 27).

Preparation of *p*H Standards

The *p*H range of culture media is covered conveniently by Sörensen's phosphate buffers.* The standards are prepared by mixing appropriate volumes of M/15 solutions of two salts, dibasic sodium phosphate (Na_2HPO_4) and potassium acid phosphate (KH_2PO_4). These salts may be obtained in the market by specifying "Sörensen purity." The M/15 solutions are prepared by dissolving 9.47 gm. of dibasic sodium phosphate and 9.08 gm. of potassium acid phosphate in distilled water, to make 1 liter of each, measured in volumetric flasks. The solutions are combined as shown in Table II.

These *p*H standards may be kept in the refrigerator for several months without any appreciable change.

Determination of *p*H by the Drop Method

The simplest means of making routine *p*H measurements is provided by a standard laboratory *p*H meter, or potentiometer. Certain of these instruments give accurate readings on samples as small as 0.5 ml. Where a *p*H meter is not available, the *p*H of colorless culture media may be determined colorimetrically by the drop method of Felton.* The standard solutions of phosphate buffers and indicators are kept in the refrigerator in glass-stoppered dropping bottles fitted with pipettes drawn out to a fine point. The indicator most commonly used in culture work is phenol red, which has a convenient color range between *p*H 6.8 and *p*H 8.4. One of the dropping bottles should therefore contain a 0.01 per cent solution of this dye in 25 per cent alcohol.

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* Sörensen, S. P. L. *Ergebn. Physiol.*, 1912, *12*, 393.

TABLE II.

M/15 PHOSPHATE MIXTURES

*p*H	M/15 Na_2HPO_4 (*ml.*)	M/15 KH_2PO_4 (*ml.*)
5.0	1.0	99.0
5.2	2.0	98.0
5.4	3.0	97.0
5.6	5.0	95.0
5.8	7.8	92.2
6.0	12.0	88.0
6.2	18.5	81.5
6.4	26.5	73.5
6.6	37.5	62.5
6.8	50.0	50.0
7.0	61.1	38.9
7.2	71.5	28.5
7.4	80.4	19.6
7.6	86.8	13.2
7.8	91.4	8.6
8.0	94.5	5.5
8.2	96.5	3.5

In determining the *p*H of an unknown fluid, one first places on a clean spot plate individual drops of the several buffer solutions corresponding most closely in *p*H to the supposed *p*H of the fluid to be tested. A drop of phenol red is added to each. Then, in close proximity to the various buffers, a drop of the fluid to be tested is mixed with a drop of the dye. The *p*H is determined by comparing the color tints of the standard buffer mixtures with that of the unknown. The *p*H of solutions con-

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Felton, L. D. *J. Biol. Chem.*, 1921, *46*, 299.

taining bicarbonate changes on exposure to air. If, therefore, a satisfactory comparison has not been made within fifteen seconds, it should be repeated with a fresh drop of experimental fluid. Theoretically, the drops of indicator and of fluid to be tested should be of the same size. For this reason, care should be taken to let the drops fall from pipettes held in a vertical position.

STANDARD FLASKS FOR USE IN DETERMINING THE *p*H OF CULTURES

When cultures in Carrel flasks are grown in a medium containing a total concentration of 0.001 to 0.005 per cent phenol red, it is possible to determine the approximate *p*H of the medium at any moment merely by comparing the culture flasks with a set of standard flasks made up with phosphate buffers containing the same indicator in the same concentration. It is necessary that the standard flasks be of the same size and contain about the same amount of fluid (1.5 ml.) as the culture flasks.

The standard flasks are made up with one-half of the required amount of fluid consisting of the standard buffers (*p*H 6.4 to *p*H 8.2), and the other half of 0.01 per cent phenol red, to make the final concentration of the indicator 0.005 per cent. But unlike the phenol red used in the drop method, the indicator employed here and in the actual cultures is the sodium salt of the dye and is prepared in the following manner:

One hundred milligrams of phenol red* are placed in a mortar and ground with 2.82 ml. of exactly N/10 sodium hydroxide, until the dye is completely dissolved. The solution is then rinsed with distilled water into a 100 ml. volumetric flask and is finally made up to volume. This makes a 0.1 per cent solution. For concentrations of phenol red higher or lower than 0.1 per cent, both the dye and the alkali are varied in proportionate amounts.

The solution of phenol red and the phosphate buffers are sterilized by heating for thirty minutes in a boiling water-bath, before the standards are made up in the culture flasks.

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* Hynson, Westcott & Dunning, Inc.

Then, when the solutions have been placed in sterile flasks, the latter are tightly closed with sterile rubber stoppers (the ends of which are cut off flush with the necks of the flasks) and hermetically sealed with waterproof cement.*

CRYOSCOPIC (FREEZING-POINT) METHOD OF DETERMINING OSMOTIC PRESSURE

In the preparation of culture media, it is a matter of great importance to keep them approximately homogeneous in salt content with the tissues and hence with the serum of the animal from which the tissues are taken. Cells are so organized that their food must be brought to them in solution. The transportation of materials in solution depends upon diffusion, and diffusion is very closely related to osmotic pressure. If cells are maintained in a medium with a greater osmotic pressure than their own contents, they tend to shrink. If the osmotic pressure of the surrounding medium is too low, they swell and burst.

Solutions with the same osmotic pressure have a number of properties in common and are said to be isotonic. Although the osmotic pressure of a solution may be measured directly, it is usually determined indirectly by measurements of vapor pressure, boiling point or freezing point, all of which properties are possessed by isotonic solutions to the same relative degree. Of these, the determination of the lowering of the freezing point is the most satisfactory method. The presence of corpuscles, colloidal suspensions and emulsions has little or no effect on the results. Hence, the determinations can be made not only on blood plasma and blood serum but also on whole blood. The osmotic pressure of Tyrode's solution, as interpreted by its freezing point (−0.62° C.), lies within the range of most normal sera. The freezing point of chicken serum usually varies from −0.60° to −0.65° C., according to the individual. Rabbit serum has a lowering of from −0.55° to −0.66° C.; human serum, from −0.48 to −0.65° C. If, in addition to Tyrode's solution, however, other substances are used in the medium, the osmotic pressure of the final mixture must be kept isotonic with the serum of the species providing the tissue. A hypotonic solution is made isotonic with a particular serum by adding a calculated amount of Ringer's solution of five times the normal concentration. If the solution is found to be hypertonic, distilled water is added.

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* A satisfactory cement for this purpose is Du Pont's clear airplane dope. No. 5332.

The apparatus used in making these determinations consists of a battery jar containing a mixture of rock salt and ice shavings. In this is placed a large test-tube (22 × 100 mm.) within which is a smaller test-tube (14 × 100 mm.) for the accommodation of the thermometer and the solution to be tested. Perforated stoppers are inserted in such a way that the thermometer does not touch the smaller tube and the smaller tube does not touch the larger tube. The air jacket between the two tubes allows the test-fluids to be cooled at a more uniform rate. A satisfactory thermometer has a range of from +1.0° to −5.0° C., with graduations of 0.01° C. A Beckmann differential thermometer may be employed, but because its mercury adjustment is easily upset, it must be checked each time before being used. Nichrome or chromel wire wound loosely around the thermometer in the form of a spiral, with six or seven turns, serves as a stirrer to keep the temperature of the solution uniform. The stirrer is admitted through a hole in the inner stopper.

About 2 ml. of the solution to be tested are placed in the inner tube. This amount should cover the bulb of the thermometer. As soon as the thermometer is in place, the stirrer is manipulated with a slow up and down motion (about 120 strokes per minute) and the course of the mercury column is carefully observed. The mercury will gradually fall below the actual freezing point because of supercooling. This fall will be followed by a sudden rise. The point at which the mercury rests for a time after this sudden rise is the freezing point. Finally, the temperature drops again.

Chapter VI

PREPARATION OF PLASMA AND SERUM

Blood plasma, that is, whole blood minus the corpuscles, provides both a nutritive substrate and a supporting structure for many types of cultures. For uniform results, plasma should be prepared from blood taken without the use of an anticoagulant. Blood serum (plasma minus fibrinogen) may be used, with or without other nutritive substances, in any desired concentration either as the entire medium or as the fluid phase of a medium consisting partly of a plasma coagulum. When serum is required, it is often prepared from plasma rather than from whole blood. This procedure eliminates the danger of hemolysis. Plasma prepared from blood that has been drawn without an anticoagulant tends to coagulate spontaneously if allowed to stand for a time in plain glass tubes. Mammalian plasma, and chicken plasma drawn with a syringe, coagulate almost immediately under these conditions and must, therefore, be prepared from blood collected in tubes lined with a thin layer of paraffin. Chicken plasma that is taken from the carotid with a glass cannula, without an anticoagulant, may be prepared from blood drawn in plain glass tubes, provided it is transferred to paraffined tubes for final storage. Both plasma and serum may be stored indefinitely after they have been dried from the frozen state [111, 136, 137] (see p. 114).

Preparation of Paraffined Tubes for the Storage of Blood Plasma

Cork stoppers to fit the tubes to be paraffined are packed for sterilization in such a way that they may later be removed from their container one by one with the fingers, without touching

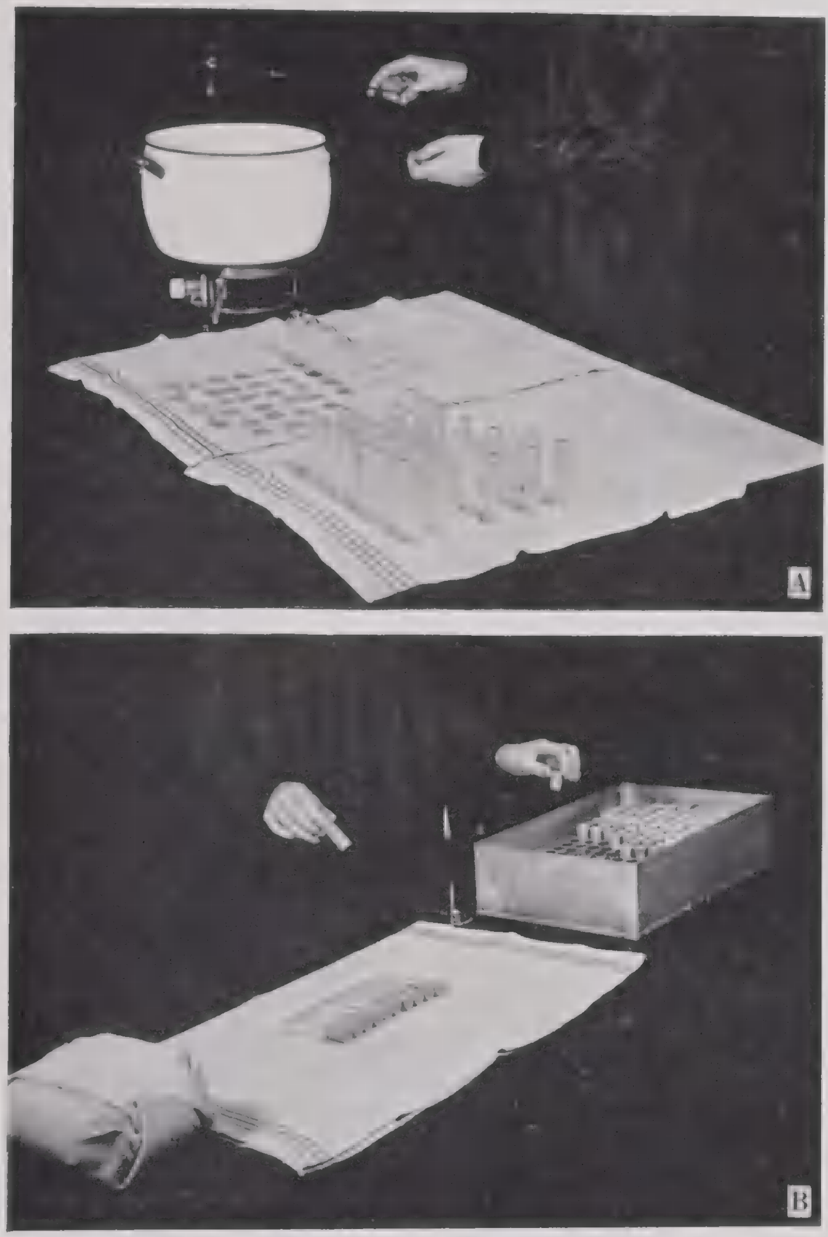

FIG. 28. *A*. Procedure followed in paraffining tubes for the storage of blood plasma. The tubes are first drained over the paraffin bath in which they have been sterilized, then set out on sterile towels and transferred from place to place until the excess paraffin has flowed out and the remainder has congealed.

B. Procedure followed in corking and wrapping the paraffined tubes. The corks are sterilized in a metal box fitted with perforated, removable racks. The paraffin at the rim is melted slightly in the flame before the cork is inserted in the tube.

any of those that remain. For this purpose, a large box of sheet metal (without a lid) is fitted with a set of removable trays, each of which is perforated with a series of holes just large enough to receive the base of the cork and spaced far enough apart to allow each cork to be removed separately as required (Fig. 28*B*). The box is wrapped with two layers of paper and sterilized by dry heat. A pair of long test-tube forceps (10 to 12 ins. in length) are also wrapped in paper and sterilized by dry heat. Six or more cotton hand towels are wrapped in protective layers of cloth and sterilized by autoclaving.

A quantity of paraffin (melting point, 56° to 58° C.), the amount depending upon the number of tubes to be prepared, is melted in a large, covered, enamel container. The medium tubes (thin glass, 18 × 100 mm.) are submerged in the paraffin in such a manner that no air will be trapped within them. The container, with heavy wrapping paper tied over the top, is then sterilized in the hot-air oven for one hour at 160° C.

After the paraffin bath has cooled to the point where it is no longer fuming, it is placed on an electric stove located on a table in a dustproof room. Sterile towels are spread out elsewhere on the same table. With long forceps, the tubes are then removed from the melted paraffin, drained over the container and allowed to stand, open end down, on a sterile towel, for further draining (Fig. 28*A*). As soon as the paraffin coating begins to congeal, the tubes are transferred to an unused part of the towel. By the time all of them have been taken out of the bath, those earliest removed will be hard enough to cork. But first, to prevent particles of paraffin from breaking off and falling into the tube, the rim of each is heated very slightly over a small flame. At no time during these operations should the tubes be held open end up, in a vertical position. After the tubes have been corked, they are wrapped in two thicknesses of sterile towels (Fig. 28*B*), for storage in the refrigerator.

Preparation of Adult Chicken Plasma

Plasma from the adult chicken has a great advantage over plasma from various other laboratory animals in that it forms a very clear, remarkably solid coagulum, even when diluted to only one-eighth or one-tenth of the medium. As a rule, the plasma obtained from other sources is either too opaque for good optical work or else it fails to produce solid clots upon coagulation.

Depending upon the amount of plasma or of serum required, one of two procedures may be employed in drawing the blood: (1) bleeding from the carotid artery for large quantities; or (2) bleeding from the wing vein for small amounts. In the first instance, it is desirable to bleed the chicken to death at one operation. It is suggested that cockerels be used exclusively for this purpose; since they are larger than pullets, they give a greater yield, their vessels are easier to locate, and, being more hardy, they are less likely to die under anesthesia. They should not be more than one year of age. To reduce the serum lipoids, they should be starved for about thirty-six hours before they are bled. Cockerels bled from the carotid artery give from 140 to 160 ml. of blood. The plasma yield varies from 40 to 60 per cent of the whole blood. Generally, over 95 per cent of the plasma may be recovered as serum (see p. 102).

In the second instance, when relatively small amounts of plasma or serum are required at infrequent intervals, or when it is necessary to have a constant supply of plasma or serum from a given chicken, it is advisable to bleed from a wing vein. From 20 to 25 ml. of blood can be taken from a chicken as often as once a week without seriously impairing its health.

The details of these alternatives follow:

Bleeding from the Carotid Artery

MATERIALS REQUIRED

(a) *Articles packed in a dressing drum for sterilization in the autoclave* (See p. 62):

Mason jar, containing irrigator (large rubber bulb with special irrigating pipette attached) and glucosol solution
2 small enamel bowls (for glucosol and Dakin's solution) wrapped in unbleached muslin
gauze sponges and pads (packed separately in muslin bags)

neck sheet for chicken, of black sateen, 1 × 1½ yds., with narrow, oval opening in center, about 5 ins. long
100 ml. beaker for tincture of iodine
4 hand towels

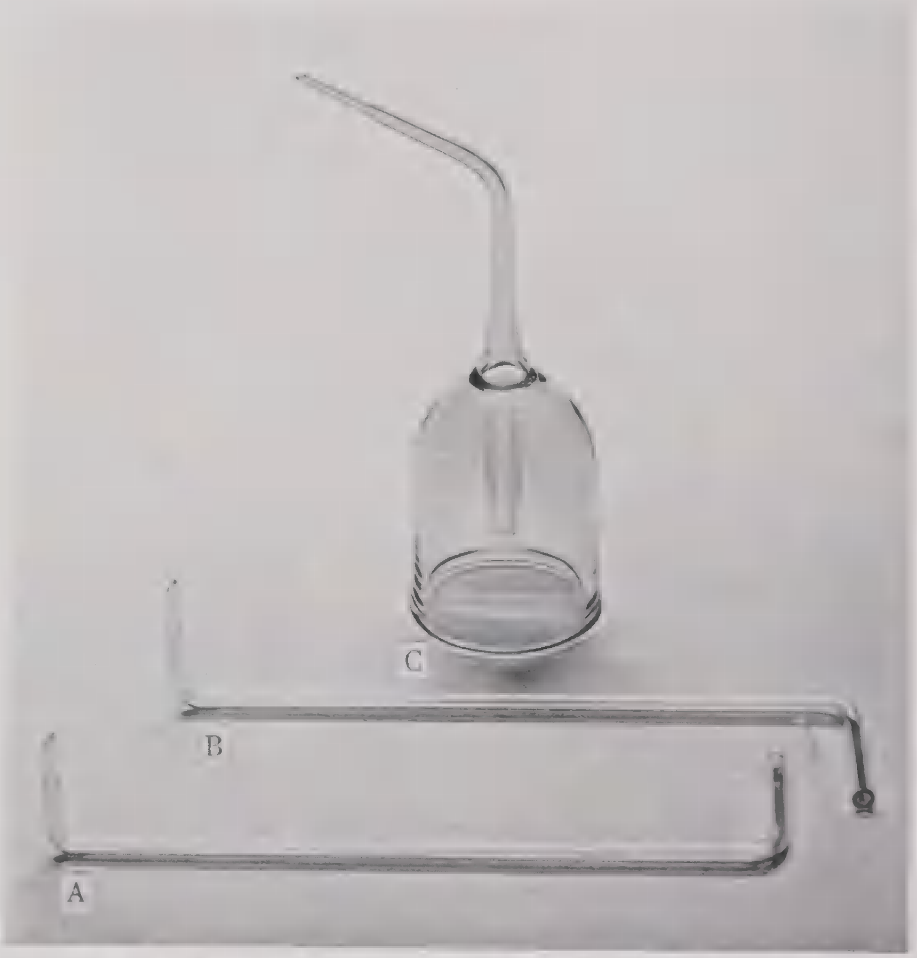

FIG. 29. Glass bleeding cannulae: (*A*) and (*B*) cannulae used in bleeding large animals from the carotid artery or from the jugular vein when filtered serum is prepared; (*C*) bell-cannula used in bleeding chickens from the carotid artery for plasma (Compare Fig. 30). For complete description, see text.

2 table covers (black sateen)
scrub-up bag (at top of drum) containing small hand towels, orange sticks, cloth cover for scrub-up table, large enamel bowl for antiseptic, soap dish and glass tray for brushes and orange sticks

(b) *Instruments packed in a metal box for sterilization by dry heat.* (They are arranged layer upon layer in order of use; each layer is protected with a black sateen pad filled with nonabsorbent cotton. The box is wrapped with paper before sterilization—see p. 65):

curved suture needles
suture silk (e.g., Deknatel, Type B, Size 2-0, cut in 4-in. lengths)
1 pr. medium-sized scissors for cutting sutures
2 pr. very fine, straight scissors for cutting into the vessel
2 very small Dieffenbach's serrefines; swivel construction; straight (in gauze sponge)
1 pr. fine, straight forceps
1 pr. fine, curved forceps
1 pr. curved, mosquito forceps
1 pr. straight, mosquito forceps
6 hemostats
2 scalpels
4 pr. towel forceps
1 pr. sponge forceps holding sponge for iodine (at top of box)

(c) *Glass bell-cannulae:*

Although various types of metal cannulae are on the market, still better ones can be blown from glass. A simple cannula of this sort (Fig. 29C) was devised by Carrel. The tip is very fine, smooth, and ground off at an angle. Just back of the tip, there is a slight constriction in the glass, at which point a ligature may be applied after the cannula has been inserted in the vessel. The cannula is about 10 cm. long. Its outlet is surrounded by a bell-like hood large enough to cover completely the mouth of the bleeding tube and, thereby, to furnish protection from falling dust particles. For sterilization, the cannulae are enclosed in gauze pads and packed over gauze-covered cotton pads in a metal container.

(d) *Bleeding tubes:*

Measuring 18×100 mm. and fitted with cork stoppers, these tubes are wrapped in paper, in lots of four or five, and sterilized by dry heat. Before the bleeding is started, they are set upright in a bowl of cracked ice. These tubes are not paraffined. The length of the tubes is important: when they are packed in ice for centrifugation, they should extend far enough beyond the carrier of the centrifuge head to protect their tops from water.

(e) *Paraffined storage tubes for plasma:*

Paraffined storage tubes are set upright in a separate bowl of cracked ice. They are described fully on page 88.

(f) *Two additional bowls of cracked ice:*

One bowl is provided to receive the filled bleeding tubes, the other to provide ice for packing the tubes in the centrifuge carriers.

(g) *Chicken board:*

A board measuring about 13×17 ins. with canoe cleats at the corners, is ready for use in attaching the wings and legs. Each wing is held down with a sand bag (about 5×14 ins., weighing from 6 to 7 lbs).

PROCEDURE

Two tables are used: The chicken is placed on one, the instruments on the other. As soon as the operator has put on a sterile mask, the dressing drum, instrument box and other materials are set out, as well as iodine, glucosol solution and Dakin's solution. The scrub-up materials are removed from the dressing drum and placed in the scrub-up room. The operator then cleanses his hands in the usual way (see p. 70), puts on a sterile gown, spreads a sterile cloth on the instrument table and arranges the instruments.

In the meantime, the chicken, ventral side up, has been strapped to the operating board by the anaesthetist, and the feathers have been plucked from the neck and upper breast region. It is then covered securely with a towel and transferred to the operating room, where it is etherized very slightly (from a small metal cone), just enough to keep it fairly quiet. Whenever possible the operator should have three assistants: one to anaesthetize and to support the neck of the chicken, one to handle the bleeding tubes, and one to centrifuge the blood as it is taken and to draw off the plasma. The bleeding can be accomplished with fewer assistants, of course, but under these conditions it takes considerably longer.

As soon as the chicken is ready the operator swabs the plucked area with iodine and arranges the neck sheet so that the opening lies over the place of incision. Each end of this opening is clamped to the skin with towel forceps. A medium, ventral incision is made through the skin of the neck with one of the scalpels, and the skin is clamped back to the neck sheet with hemostats. A median incision is then made through the subcutaneous tissue with the second scalpel. With the curved mosquito forceps and the fine, straight forceps, one of the carotid arteries is now freed from the surrounding musculature and all adherent subcutaneous connective tissue. (The carotid arteries, of which there are two, lying side by side, are fairly deep.) The freed portion of the artery should be about 4 cm. in length. The distal end is now ligated and the proximal end clamped with a serrefine. After the vessel has been cleansed with glucosol solution by means of the irrigator, a small, clean incision is made in its wall midway between the serrefine and the ligature. The serrefine is then opened for a moment to enable the blood to flow freely and to flush out any clots that may have formed between the serrefine and the incision. These clots, containing traces of tissue juice from injured cells, may, if collected in the first tube, induce further coagulation. Before the glass cannula is inserted, the incision is thoroughly washed with glucosol solution, and a sterile gauze pad, with a slit-like opening, is placed over the field of operation. If necessary, a ligature may be applied to hold the cannula in place.

The blood is now collected in bleeding tubes that are held under the bell of the cannula until they are about three-quarters full (Fig. 30). After filling each tube, the outflow may be controlled by means of the serrefine, or preferably by press-ing the forefinger on the vessel, so as not to produce injury. For a long bleeding, e.g., sixteen to eighteen tubes, it is generally wise to change the cannula, especially if the blood begins to come slowly.

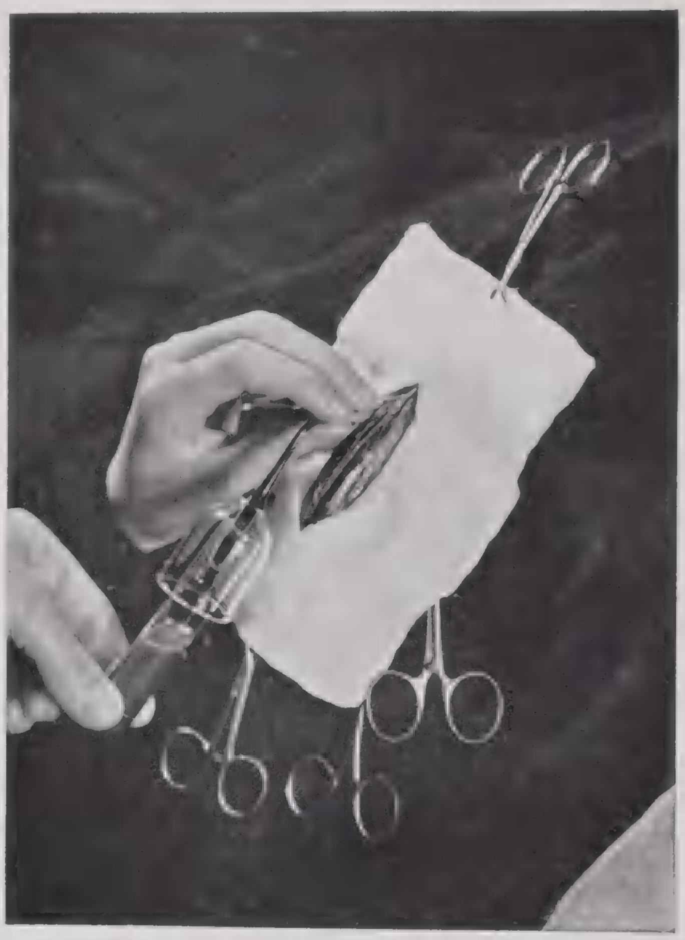

FIG. 30. Glass bell-cannula used in bleeding a chicken from the carotid artery. The apron protects the mouth of the bleeding-tube from falling dust particles.

As soon as a few tubes of blood have been withdrawn, they are centrifuged in ice-packed carriers for six minutes at 3000 r.p.m., or for seven minutes at 2800 r.p.m. After centrifugation, the clear, supernatant plasma is withdrawn with a large bulb pipette (Fig. 31) capable of holding 100 ml. This pipette is fitted with a mouth-tube and operated by suction. In removing the plasma, extreme care is taken not to disturb the underlying blood cells. The plasma from five or six tubes may be drawn off at one filling and then distributed into the paraffined storage tubes, with about 6 ml. in each. Before being stored in the refrigerator, these tubes are labeled with the chicken's number and the date.

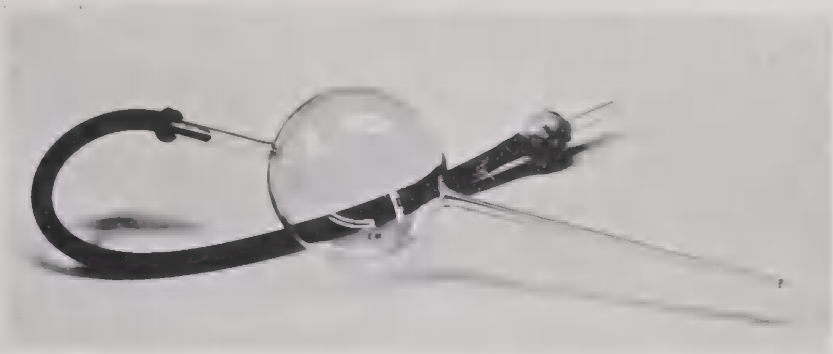

FIG. 31. Large-bulb pipette used in transferring plasma from bleeding tubes to paraffined storage tubes.

If any part of the plasma that has been prepared is to be processed for serum, the plasma is pipetted into unparaffined tubes, a drop or two of embryo tissue juice is added to each, and the tubes are left in the incubator overnight to ensure complete coagulation (see p. 102).

If the chicken has been only partially bled, and is to be used again, a ligature is placed at the proximal end of the artery, just above the incision, and the serrefine is removed. After all of the exposed tissues have been thoroughly washed with glucosol solution, the outer skin is brought back into place and sutured. A few weeks later, the neck may again be opened and the animal bled to death from the other carotid.

Bleeding from the Wing

The blood is withdrawn by means of a 20-ml. syringe and 2-inch Luer needle (gauge No. 18 or 19) previously sterilized in dry heat and cooled in the refrigerator. After a few feathers have been plucked from the inner part of the wing, the bird is laid on its side and the wing is supported on a sand-bag. The surface is swabbed with iodine and the needle inserted into the large vein quickly, with the least possible degree of injury to the tissues, against the direction of flow, that is, towards the tip of the wing. In withdrawing the blood, the plunger must not be pulled with too much force. If the needle has been properly inserted, the blood will flow freely into the syringe.

When the desired amount of blood has been taken, the needle is removed from the syringe and the blood injected slowly into chilled paraffined storage tubes. The tubes are then centrifuged in ice-packed carriers for four and one-half minutes at 3000 r.p.m. After centrifugation, the clear supernatant plasma is transferred to fresh tubes. These tubes must be paraffined and thoroughly chilled, if the yield is to be retained in the form of plasma.

PREPARATION OF CHICKEN PLASMA WITH AN ANTICOAGULANT

For certain types of experiments it is by no means possible to use plasma containing an anticoagulant. For some experiments plasma containing an anticoagulant may be used if thrombin is added to the medium. Unfortunately, however, the presence of an anticoagulant seems to render different samples of plasma from the same batch somewhat irregular in their clotting time. This irregularity may perhaps be due to an unequal distribution of the anticoagulant. But for many general purposes, and where quantitative growth measurements are not required, the presence of a modest amount of anticoagulant makes little difference provided the culture medium contains a considerable quantity of tissue extract and does not contain substances that inhibit the clotting action.

When heparin is used in bleeding a fowl for plasma, sufficient heparin for 100 ml. of blood (e.g., 1 ml. of glucosol containing 1 mg. of heparin) is placed in a graduated 250-ml. centrifuge bottle fitted with a glass filling bell and a glass or metal cap, as shown in Fig. 32. (At the time of bleeding, the protective cap is removed from the tube of the filling bell and the bleeding cannula, with its own bell-like apron, is inserted.) A second centrifuge bottle, without heparin, is prepared in the same manner for whatever blood is drawn in excess of 100 ml. If 100 ml. of blood is collected in the first container, the plasma that is prepared from it will therefore contain a known amount of heparin (i.e., 1:100,000). The blood that is collected in the second container, without heparin, may be allowed to coagulate in the usual manner for the preparation of serum (see p. 102). As an alternative, the balance of the yield may be collected in plain glass tubes and centrifuged in ice-packed carriers for the preparation of plain plasma without heparin.

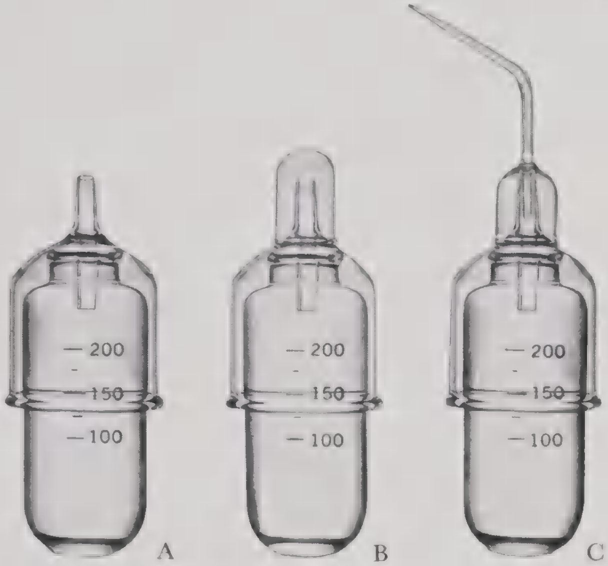

FIG. 32. Device used for bleeding from the carotid artery of a chicken directly into a 250-ml. centrifuge bottle, when an anticoagulant is present: (*A*) centrifuge bottle fitted with a large filling bell; (*B*) tube of filling bell protected by glass cap; and (*C*) glass cap removed and outlet of bleeding cannula (see Fig. 29) inserted into tube of filling bell.

If sodium citrate is used, it may be prepared as a 10 per cent solution in glucosol. For 100 ml. of blood, 2 ml. will be required.

PREPARATION OF MAMMALIAN PLASMA

Small animals (rats, mice and guinea-pigs) may be bled either from the heart or from the aorta. Unless an anticoagulant is used, however, the first means should not be employed, for when the heart is punctured, the entire yield may coagulate before centrifugation. As the needle pierces the skin, the muscles, and the mucous membranes, it collects a certain amount of tissue juice that is drawn up into the syringe along with the blood. Larger animals, such as rabbits, cats and dogs, are usually bled either from the jugular vein or from the carotid artery.

In bleeding small animals from the heart, only a small amount of ether is given. The animal is placed in a battery jar, with a piece of cotton soaked in ether. As soon as the animal is unconscious, it is removed from the jar and stretched on a board, ventral side up. The hair is clipped from the area over the heart. After the skin has been prepared, the point at which to insert the needle is determined by palpation, and the region is painted with 7 per cent tincture of iodine. Then, with a 1½-inch Luer needle (gauge No. 19), fitted to a 20-ml. Luer syringe, the skin is pierced and the needle is pressed downwards very gently, through the anterior wall of the heart. The posterior wall should not be punctured. Ordinarily the pressure of the blood flow will force back the plunger. Once the blood has been obtained, it is transferred to chilled paraffined tubes and centrifuged for three or four minutes in the manner described on page 96.

In bleeding small animals from the aorta, a median ventral incision is made in the abdomen of the anaesthetized animal, the internal organs are gently pushed aside and the aorta is exposed. For a mouse, a 2-ml. Luer syringe will be adequate; for larger animals a 10 or 20-ml. syringe is necessary. Usually, a gauge No. 23 needle, ⅝ inch long, may be used. The syringe itself must be well chilled. In addition, for such small quantities of blood, it is usually safer to use a solution of heparin. As an alternative the wall of the syringe and the piston may be lubricated with sterile olive oil of good quality. After the vessel has been exposed, it is washed with warm glucosol solution. Because of limited space, a serrefine cannot easily be used. Instead, a little pressure may be applied with the finger at the proximal end of the aorta. The distal end is clamped off with a mosquito clamp and the aorta is punctured close to the proximal end. The moment the needle has been inserted, the finger pressure is released. At first, no force should be used in withdrawing the plunger; towards the end, a very slight pressure may be necessary. The blood is placed immediately in small, well-chilled paraffined tubes, 10×75 mm., and centrifuged for two or three minutes at 3000 r.p.m.

In bleeding large animals from the jugular vein, or from the carotid artery, an incision is made, and the vessel is exposed and thoroughly washed in the manner described on page 95. If the blood is drawn with a syringe and needle, or with a fine cannula, the opening may be closed with a very fine suture. The same vessel may then be used again at some later date. Before an attempt is made to suture the vessel, the exposed portion is clamped off at each end with a soft serrefine or with soft-jawed forceps and the lumen is washed out with warm glucosol solution, with a fine-tipped irrigator. Very gentle pressure on the bulb of the irrigator will set up a sufficient flow to wash out any blood remaining in the vessel. A trace of vaseline is then applied directly over the puncture in the vessel, which is sutured with an arterial needle and suture material of the finest quality; the needles and sutures are sterilized in vaseline. When the opening has been closed, a dry sponge is placed directly over the sutured area and gentle pressure is applied with the finger. The serrefines are now removed. After a few minutes, the finger and the sponge may be withdrawn. By this time, the vessel should be free from any oozing of blood.

In bleeding large animals from the carotid artery (or from the jugular vein) for serum to be filtered before use, it is convenient to employ simple glass cannulae of the design illustrated in figures 29*A* and 29*B*. The free ends of this type of cannula is bent at right angles to its long axis to form a 90° angle with the bent end inserted in the vessel. In some of the cannulae, the free ends are bent to the right, in others, to the left. When an animal is being bled from the left side of the neck, the cannula used is that illustrated in figure 29*A*; when from the right side, the cannula chosen is of the type shown in figure 29*B*.

Human blood is usually taken from the basilic vein of the forearm but it may also be obtained from the radial artery or from the ulnar vein of the hand. The moment the syringe needle is withdrawn from the vessel, a sterile pad is pressed firmly with the finger for two or three minutes directly over the point of insertion, to prevent the formation of a hematoma. If there is any doubt about procuring the blood without coagulation, heparin should be used. Otherwise, the wall of the syringe and the piston may be lubricated with sterile olive oil. In any event, the syringe should be thoroughly chilled beforehand, and no time should be lost in transferring the blood to chilled tubes to be centrifuged in ice-packed carriers.

Human tissues for shipment should be placed in a tube containing blood plasma from the patient or donor. The plasma should be allowed to coagulate around the specimen. If plasma cannot be prepared, whole blood may be used.

Preparation of Serum

When plasma that has been prepared without an anticoagulant is allowed to coagulate, the serum may be expressed by removing the fibrin mechanically. This procedure is extremely simple for the plasma of rats, guinea-pigs, rabbits, cats, dogs and humans, which tends to coagulate spontaneously even without the addition of chick embryo extract. In these instances, the fibrin clot is broken up with a glass rod and the material centrifuged. Most of the fibrin will adhere to the glass rod during the agitation and may be withdrawn immediately. Centrifugation, however, may cause a second deposition of fibrin. If it does, the whole process is repeated until no more fibrin is brought down by centrifugation. The serum is then ready for use. It is stored in the refrigerator in tubes fitted with rubber stoppers.

In the preparation of chicken serum from coagulated plasma, the procedure is somewhat complicated by the extreme firmness of the coagulum that is formed. Furthermore, it is difficult to get rid of the last traces of fibrinogen. The simplest means of accomplishing this is to coagulate the fluid plasma completely by incubating it overnight with a drop or two of embryo extract or thrombin (see p. 172) added to each tube. The coagulated plasma is then ground in a mortar, with sterile sand. To guard against contamination from the air, the mortar and pestle should be protected by a canopy of moist, sterile cellophane (previously cut to size, folded and autoclaved in Mason jars partly filled with water). After thorough grinding, the serum is separated from the sand by centrifugation. Before being used, a small sample of the serum should be incubated with equal parts of embryo extract or with thrombin to make certain that all the fibrinogen has been removed.

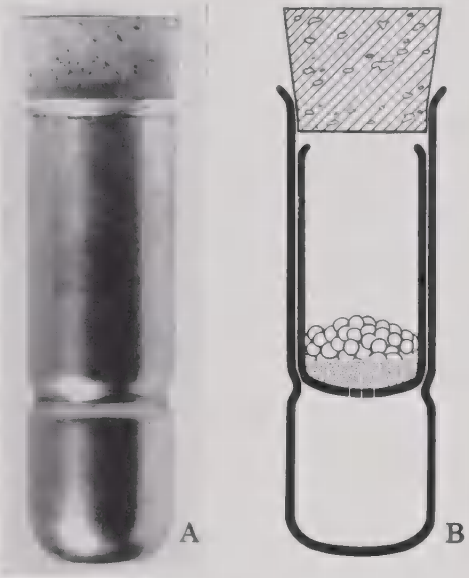

FIG. 33. Double centrifuge tube for the preparation of chicken serum from coagulated plasma: (*A*) tube constructed from sterling silver; (*B*) cross-sectional diagram. For description, see text. (C. A. Lindbergh, unpublished experiments.)

In 1933 Lindbergh* devised a simple and effective technique for obtaining the greatest amount of serum from coagulated plasma by centrifugation. This technique, which involves the use of a double centrifuge tube, is now definitely preferred in the preparation of serum from chicken plasma. Under suitable conditions coagulated chicken plasma will yield over 95 per cent of its volume in serum, with the least danger of contamination.

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* Lindbergh, C. A. Unpublished experiments.

The apparatus (Fig. 33) consists of two silver-plated or chrome-plated tubes, one of which fits loosely inside of the other. The outer tube is made with a slight constriction in its wall about two-thirds of the way down from the top. This constriction supports the inner tube, which is loose enough to be removed easily and short enough to allow the outer tube to be corked. Several holes, each approximately 0.5 mm. in diameter, are drilled in the bottom of the inner tube. These holes are covered with a layer (5 to 10 mm. thick) of fine quartz sand and are small enough to prevent the grains of sand from passing through during centrifugation. Above the quartz sand is placed a layer (6 to 8 mm. thick) of glass beads, each having a diameter of approximately 3 mm. A sufficient number of beads must be present to cut the plasma clot into fine fragments as it is forced through during centrifugation, whereas the layer of sand must

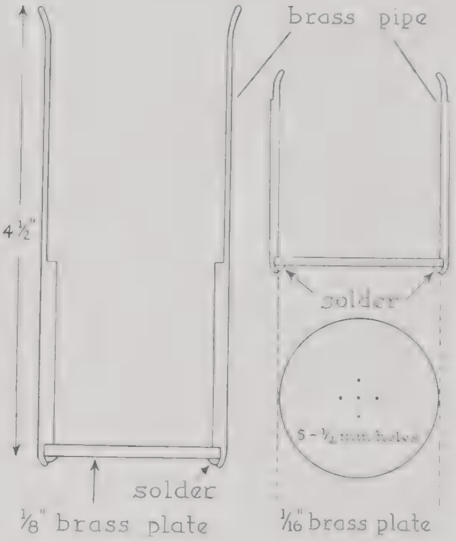

FIG. 34. Diagram showing a method of constructing a Lindbergh double centrifuge tube by boring out a section of brass pipe to receive the inner tube, also constructed from brass pipe of lesser caliber. Discs are soldered in place to provide a bottom for each section; and the whole device is chrome or silver plated. (Compare Fig. 33.)

be thick enough to prevent fibrin from passing through with the serum.

The volume of the outer tube below the constriction must not be less than the volume of coagulated plasma to be placed in the inner tube. The centrifugal force required for operation is approximately 1000 times gravity. The time required for centrifugation at 2200 r.p.m. (International centrifuge, size 2, type B, with No. 239 head) varies from seven to ten minutes, according to the size of the tubes, the centrifugal force and the amount and firmness of the coagulum. The apparatus is assembled as for use and wrapped securely in paper before sterilization. It should be sterilized in an upright position.

In the author's laboratory, the apparatus that is used was constructed from two lengths of brass pipe, one of which was bored out to permit the shorter unit, of lesser caliber, to rest, with very close tolerance, on a ledge part way down (Fig. 34). Brass discs were used to provide bottoms for the two parts; and the entire apparatus was silver plated.

When the plasma left to coagulate in plain glass tubes is firm enough, the mass is dislodged, broken up by a large pipette and transferred to the inner tube of the apparatus, which is then corked and centrifuged in one of the standard 250-ml. centrifuge cups. After centrifugation, the cork is removed, the inner tube withdrawn, and the serum pipetted from the lower compartment.

If it is not known at the time of bleeding just how much of the yield should be processed for serum, the latter may be prepared from the plasma as and when required.

If it is desired to prepare chicken serum directly from whole blood that is taken without an anticoagulant, the blood is collected in a 250-ml. centrifuge bottle (see p. 98) and allowed to stand for an hour at room temperature until coagulation is complete. After centrifugation, the serum is pipetted off and stored in tubes fitted with rubber stoppers.

Some investigators make considerable use of human placental cord serum. After the blood has been collected, it is allowed to coagulate and the serum is prepared in the usual manner.

Sterility tests are made on the individual samples, and those that are contaminated or hemolytic are discarded. As an added precaution, the remaining samples are pooled and filtered.

Tissues to be stored in the cold room or refrigerator (at 4° C.) are usually placed in 10 per cent serum (homologous, when possible) diluted with Tyrode's solution or one of the other balanced salt mixtures (see also Hanks and Wallace[127]).

CHAPTER VII

PREPARATION OF TISSUE EXTRACTS

IN 1912, when Carrel's well-known strain of connective tissue cells was in its first year of cultivation, it was discovered[33] that embryo tissue extract had remarkable powers of promoting cell growth and multiplication. Up to the time of this discovery, the subcultures were becoming smaller and smaller with each subsequent transfer. While blood plasma was adequate as a source of nutrient materials, it did not stimulate the cells to divide fast enough to compensate for the amount of tissue lost in transplanting the fragments to new media. With the addition of tissue extract, however, this situation was completely remedied, and the strain (Fig. 2) continued to multiply for thirty-four years (see p. 8) in a combination of fowl plasma and chick embryo tissue extract, diluted with Tyrode's solution.

Since Carrel's[33] early experiments there have been many attempts to determine the chemical nature of the constituents responsible for the stimulating effect of embryo extract. Baker and Carrel [10] obtained active fractions of the extract by precipitation with carbon dioxide, and found that the activity was concentrated largely in the protein portion containing nucleoprotein and glycoprotein. It was further observed[43] that proteoses and higher protein degradation products also had very potent growth-promoting properties. Because of these studies, Carrel and Baker rejected their earlier assumption that the extract contained a specific hormone essential to cell division. They suggested that growth-activating substances are not preformed in the embryo extract but are continuously made from its proteins by the cells themselves or by enzymes contained in the medium, a suggestion that was supported by the subsequent findings of Willmer and Kendal.[272] Fischer[99, 107] prepared a very active growth-promoting factor from beef embryos by isolating the nucleoproteins; but repeated reprecipitation of this material resulted in loss of potency. Growth-promoting activity appeared to be associated particularly with fractions containing predominantly nucleoproteins of the ribonucleic acid type. Fractions relatively high in desoxyribonucleic acid were much less active. Fischer's results were confirmed by Davidson and Waymouth,[70] who worked with the nucleoprotein from dried sheep embryo extract. In an extension of this work, Waymouth[262] found that the content of ribonucleic acid seemed somewhat more important than that of desoxyribonucleic acid, but no absolute correlation existed between the growth-promoting activity of the material and the relative proportions of the two types of nucleic acid. It should be added that the tentative identification of the active principle of embryo tissue extract with nucleoproteins of the ribonucleic acid type is in accord with recent views on the distribution and importance of nucleoproteins in all types of cells.[53, 237, 64]

Jacoby, Trowell and Willmer,[145] working with fibroblast-like cells, have shown that the concentration of embryo extract present in the medium is a more important factor in determining the number of mitoses produced than is the time during which it acts, and that it is not necessary for the embryo extract to be present in any appreciable amount during the actual division process. The only necessity is that the cells shall have been treated with extract ten to twelve hours previously, but it makes little difference whether the extract is applied for one or for ten hours. If the extract remains on the culture for more than ten hours it is possible for the cells produced at the first division to give rise to a second generation. It would seem, then, that if a sufficient quantity of embryo extract is present in the medium when the cell divides, the cell can divide again; if not, the cell has very little chance of undergoing a second division unless more extract is subsequently added. The same investigators have also shown[271] that embryo extract accelerates the actual process of mitosis and stimulates cell migration, thus adding to the area of the cell colony. They have also made it abundantly clear that cells do not divide just because they have reached a certain size. Cells divide only when stimulated to do so by special substances present in the medium; when these substances are absent, the cells become, and remain, larger.

Gaillard[112] has reported an interesting series of experiments on the effect of cultivating osteogenic spindle cells in the presence of extracts prepared from embryos of increasing ages. Extract from eight-day-old embryos was used as part of the original medium for both the test cultures and the controls. Every two or three days the fluid phase of the medium was changed on both sets of cultures, but whereas the composition of the medium used on the control cultures remained the same, the test cultures were treated each time with extract prepared from embryos that were two or three days older than before. As a result, the cultures that had been treated with extract from embryos of increasing ages developed histologically differentiated bone within twelve days, whereas the control cultures did not. These experiments demonstrated the manner in which progressive changes taking place in the developing embryos can affect the extracts made from them and the manner in which these extracts may affect tissues that are cultivated in them.

Carrel introduced the use of embryo tissue extract as a means of stimulating cell growth, so that more and more cultures of a homogeneous nature could be propagated for use as biological test material. When extract is used for this purpose, the age of the embryos from which it is made is of no great importance so long as its composition is reasonably uniform from batch to batch. But for those who are interested in studying the inherent abilities of certain cells to form specialized structures, the results obtained by Gaillard are most interesting.

Over the years considerable use has also been made of extracts prepared from the tissues and organs of the adult animal. In the cultivation of mammalian tissues, splenic extracts are frequently used in place of extracts made from embryos. Furthermore, in studying the specific effects of various organ secretions on growing cells, it is common practice to prepare from these organs crude extracts, or cell-free emulsions, that may be added directly to the culture medium.

Chick embryo tissue extract is used extensively to promote the growth and multiplication of every type of cell derived from embryonic and adult tissues, homologous and heterologous alike. It is also useful as an agent for hastening the coagulation of blood plasma. Its very nature, of course, precludes the possibility of universal standardization. But each individual investigator can arrive at a certain degree of uniformity in his own material by establishing a strict order of routine in caring for the eggs and preparing the extract. Then, if he ascribes a certain biological effect to the presence of a given amount of tissue extract in a medium, he is in a better position to reproduce his results at some future date when the original sample is no longer available.

It follows that if such an experiment is to be recorded in the literature, the investigator must take pains to state the age of the embryos used and the exact manner in which the extract was prepared. It is not enough to state that 10 or 20 per cent embryo tissue extract was used or incorporated in a given medium. If embryos of a certain age are chopped by hand, with scissors or knives, and if the resulting pulp is centrifuged in a pure state and diluted only in the final medium, the resulting mixture will differ appreciably from one that is prepared by allowing the pulp to stand with diluting fluid for an hour or so before centrifugation. In the latter instance, certain water-soluble substances will be transferred from the pulp to the diluting fluid. Similarly, embryos that are chopped by hand will provide an extract that is definitely inferior to extract prepared by means of an efficient motor-driven tissue homogenizer. The homogenizer actually disrupts the cells, whereas hand-chopping provides at best an uneven mass of tissue clumps and cell aggregates.

In the preparation of chick embryo tissue extract, it is of the utmost importance that the eggs show a high percentage of fertility; that is to say, they should be of the grade sold expressly for breeding purposes. Under no conditions should they be procured by random selection in the public market. Until ready for incubation, these eggs are stored in a cool room or refrigerator. But they must not be allowed to freeze. The temperature chosen for incubation should lie between 37° and 39°C. Plenty of moisture should be present in the incubator; and both the incubator and the eggs should be well ventilated. Wire racks provide more adequate ventilation than paper trays.

It is recommended that chick embryo extract be made from ten or eleven-day-old embryos. In removing the embryo from the egg, the shell is first cracked very slightly on one side with a suitable metal object. Then, while the operator holds the egg in an upright position over a waste pan, the shell is completely removed from the upper half of the egg in such a manner that the escaping fluid is allowed to pour off over the side of the lower portion without displacing the embryo. The embryo may now be grasped around the neck with a pair of curved forceps and removed to a sterile container. The extract is prepared according to one of the following procedures.

Extracts Prepared in a Motor-driven Pyrex Homogenizer

According to this procedure, the embryos and a measured quantity of Tyrode's solution or its equivalent (e.g., 2.0 ml. per embryo) are ground in a mechanical homogenizer consisting of a toothed, motor-driven glass pestle that fits closely into a large, graduated Pyrex test-tube. After centrifugation (ten minutes at 2500 r.p.m.), the material is further diluted to give an extract consisting of one part tissue and ten to twenty parts diluent. This is the method of choice.

In 1922 Hagan[*] devised a simple tissue homogenizer from two test-tubes, one of which fitted closely into the other. In 1931 TenBroeck[†] improved this device by using heavier glass and providing for greater ease of manipulation. Glaser and Coria[‡] elongated the pestle by drawing it out to a tube that could be attached to a motor. And Potter and Elvehjem* added tooth-like protrusions to the end of the pestle.

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[*] Hagan, W. A. *J. Exp. Med.*, 1922, *36*, 711.

[†] TenBroeck, C. *Science*, 1931, *74*, 98.

[‡] Glaser, R. W., and Coria, N. A. *Am. J. Hyg.*, 1935, *21*, 111.

In the author's laboratory a large Pyrex homogenizer (Fig. 35) has been devised in which TenBroeck's design has been used for the outer tube, and Potter and Elvehjem's design for

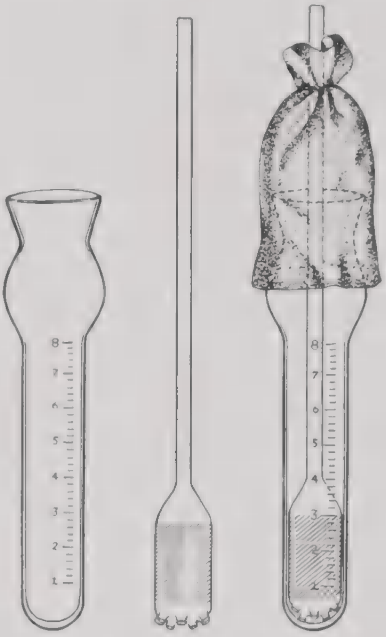

FIG. 35. Pyrex homogenizer for use in preparing embryo tissue extracts. For complete description, see text.

the pestle. The outer tube has an outward flare at the top to facilitate loading. Just below the flared portion, there is an enlarged section that serves to lessen the danger of the tissues and diluent overflowing during the grinding. Below the enlargement the tube has a capacity of 85 ml. when the pestle is removed; with the pestle in place, its capacity is 45 ml. The pestle rod is made from a piece of 8-mm. capillary tubing and is about 230 mm. in length. One end is sealed to a thick-walled cylindrical bulb, which is about 65 mm. in length and rounded at the tip to fit the bottom of the outer tube. The sides of the bulb are straight, and parallel to the sides of the outer tube for a space of about 40 mm. Twelve or more tooth-like protrusions are fused into the tip of the pestle bulb and ground down until their edges are sharp. The clearance between the pestle bulb and the outer tube is so slight that the pestle, falling by its own weight into the tube, when both are dry, descends very slowly. The pestle is rotated by a cone-drive stirring motor at a speed of 80 to 90 r.p.m. To reduce the danger of breakage a short length of rubber tubing is used to connect the pestle with a glass chuck attached to the stirrer.

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* Potter, V. R., and Elvehjem, C. A. *J. Biol. Chem.*, 1936, *114*, 495.

During the grinding process, the tube is moved up and down while the pestle is revolving. The tissue is torn apart by the protrusions on the end of the pestle, and the fragments remain below the pestle bulb until they are small enough to pass along the straight sides of the bulb, where additional grinding takes place. To protect the material from bacterial contamination during the grinding, a canopy of Fiberglas cloth (sewn with glass suture material) is arranged over the mouth of the tube. The canopy, which is sterilized with the apparatus and can be cleaned in acid, fits closely around the pestle but is not permitted to rotate with it. Six to eight embryos may be processed at one time.

Extracts Prepared from Hand-chopped Tissues

According to this procedure, which is recommended only when a mechanical homogenizer is not available, the embryos are deposited in a large, sterile watch glass supported on a smaller, open Petri dish and are chopped to extreme fineness with curved scissors. During the chopping, they are protected from falling dust particles by a large Petri dish cover that is held above them. A coarse-tipped pipette is used to transfer the tissue pulp to the centrifuge tubes. The pulp is diluted 1:4 with Tyrode's solution (or its equivalent) and allowed to stand for an hour before centrifugation (ten minutes at 2500 r.p.m.).

The extract is stored in the refrigerator in Pyrex tubes fitted with rubber stoppers. Before being used, the material is frozen twice with carbon-dioxide ice and allowed to thaw out between the two freezings. This treatment reduces to a minimum the danger of having living cells in the finished product.

Embryo extract from other species (e.g., rats, guinea-pigs and rabbits) is prepared in a similar manner. The gravid uterus is removed aseptically and placed in a sterile Petri dish. The embryos are then removed one at a time by cutting through the uterine wall and separating them from the placenta.

When it is necessary to prepare large quantities of extract from adult tissues that are unusually fibrous and tough, the material is sometimes passed through a grinding device of the type designed by Latapie.* With this apparatus, tissue fragments are fed into a cylinder through an opening in the top and are gradually forced against cutting discs by turning a hand wheel. The discs are operated by means of a hand crank or motor.

Hetherington and Craig[138] reported the use of chick embryo extract that had been stored for long periods after the extract was dried from the frozen state. Several procedures for drying from the frozen state have been described under the terms "Lyophile," "Cryochem," "Adtevac" and "Desivac" processes.[111] In each of these processes the material is frozen and then desiccated in vacuum at a rate sufficient to maintain freezing by continued cooling incident to loss of latent heat of vaporization. The processes differ essentially in the means used to remove the water evaporated from the material undergoing desiccation.

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* Supplied by Arthur H. Thomas & Co., Philadelphia.

Chapter VIII

DEVELOPMENT OF SYNTHETIC MEDIA

By Joseph F. Morgan, Ph.D.

ONE of the most important undertakings in the field of tissue culture has been the constant attempt to devise an adequate medium of chemically known constitution. The advantages to be gained from such an achievement are great. Not only would a means be provided for making accurate and precise measurements of the exchange of substances between cells and medium, but a method would be furnished for reproducing at any time the exact conditions of a given experiment, conditions which could be subjected to accurate analysis. Once a synthetic medium has been devised, it should be possible to determine exactly what substances are required by the cells as essential nutrients, and to discover any nutritional differences that may exist between cells and tissues of various types, and between normal and malignant cells. If such differences could be established, the use of antimetabolites might offer a promising means of controlling the growth of abnormal cells.

Nutritive media employed for the growth of tissue cultures have been derived, directly or indirectly, from the organism, and consist of blood plasma, blood serum, embryo tissue extract, body exudates, and extracts of various tissues and organs. The complexity of these natural substrates has necessitated the employment of two general methods of approach in attempts to devise a synthetic medium. The first method, which is analytic, involves the breakdown of complex naturally-occurring materials in an effort to determine the essential constituents. The second method is directed towards the building-up of a satisfactory growth medium by the use of various combinations of chemically-pure ingredients, including amino acids, vitamins, lipids, intermediary metabolites, and accessory growth factors. This latter method is greatly facilitated by the wealth of biochemical information now available on the nutritional requirements of man, of animals, and of microorganisms.

Lewis and Lewis[162] were among the first to attempt to study the behavior of cells in media of known composition. In 1911 they investigated the survival of chick embryo tissues in solutions containing varying concentrations of sodium chloride, calcium chloride, potassium chloride, and sodium bicarbonate, and found that growth was influenced to a marked degree by variations in the proportions of these salts. They also found that excellent growth could be obtained in Locke's solution containing combinations of amino acids and polypeptides of known composition, and that media containing dextrose or maltose were better than pure salt solutions. Unfortunately no precise details of these media were supplied. Later work by Burrows and Neymann[30] indicated that hydrolytic products of egg yolk, and mixtures of ten amino acids were toxic for embryo chick cells. The concept that amino acids are toxic for tissue cultures has persisted until comparatively recent times,[103] although the results of Burrows and Neymann may possibly be attributed to the use of unphysiological concentrations or to the lack of accessory growth substances in their media.

The discovery by Carrel[33] in 1913 that extracts of tissues, and particularly of embryonic tissues, greatly accelerated the growth of fibroblasts *in vitro* naturally focussed attention upon the role of proteins in cell nutrition. Thus, Carrel and Baker[43] discovered that fibroblasts, epithelial cells, and monocytes, when cultivated in plasma, could obtain their nitrogen from proteoses and possibly other primary derivatives of a variety of proteins. They also found that peptic digests of proteins, containing large amounts of proteoses and peptones, stimulated the cells to greater activity than did tryptic digests, which consisted almost entirely of lower protein split-products. Commercial peptones in general gave variable results, although Witte's peptone appeared suitable. Fractionation of the proteoses of Witte's peptone revealed that their growth-promoting activity was distributed among several fractions.[12] Furthermore, the stimulating quality of the proteoses was attributed to a general effect upon cell nutrition and not to any single constituent of hormonal nature. Baker and Carrel[11] further established that pepsin hydrolytic products of crystalline egg albumin, purified casein, crystalline edestin, and purified fibrin are utilized by fibroblasts for their proliferation. Stimulation was actually due, therefore, to hydrolytic products and not to impurities present in their earlier digests. Preliminary denaturation of the proteins did not appear to alter the nutritive properties of the subsequent hydrolyzates.

In 1928 Baker and Carrel[11] studied Crocker 10 sarcomatous fibroblasts of the rat grown in liver digests that had been hydrolyzed by pepsin, by trypsin, by erepsin, and by hydrochloric acid, and concluded that proteoses were utilized but were not required for growth. Peptones, peptides, and amino acids were also utilized. These findings pointed to a nutritional difference between normal and malignant cells, since normal fibroblasts could not utilize peptides and amino acids for growth. Carrying this work further, Baker[7] found that sarcoma cultures, grown for forty-five days in a medium composed of chicken plasma, peptic digest of casein, glycine, thymus nucleic acid, ash of liver, glutathione, and hemoglobin behaved almost as well as sister cultures grown in plasma and embryo extract. This medium, however, would not support the growth of normal fibroblasts.

The results of Baker and Carrel warrant the conclusion that protein degradation products alone do not provide an adequate diet for all types of cells. The presence of plasma, embryo extract, or serum is also necessary. This supplementary action of complex natural substances has been attributed by Carrel and Baker[43] and by Willmer and Kendal[272] to the presence of enzymes that slowly digest the proteoses to utilizable amino acids.

In 1933 Vogelaar and Erlichman[254] reported a feeding solution that consisted of irradiated beef plasma, together with Witte's peptone, hemin, cystine, insulin, thyroxine, glucose, and the usual salts. In this medium they were able to keep a strain of fibroblasts from the human thyroid gland in a state of active proliferation for a period of three months. Later[86] sheep plasma was substituted for beef plasma, and the period of cultivation was extended to one year. The incorporation of insulin in this mixture was found to result in a marked improvement in the general appearance of human fibroblast cultures, and these authors considered it to be an important constituent of any artificial feeding solution containing glucose. It was further suggested that this medium might be improved by the addition of vitamins and additional hormones.

Using the mixture of Vogelaar and Erlichman as a basis, Baker[8] developed a feeding solution for fibroblasts and epithelial cells. In addition to all the substances used by Vogelaar and Erlichman, this mixture contained a number of other constituents that were found to enhance its power to maintain cell life and promote growth. These additional substances consisted of vitamin A (containing some vitamin D), ascorbic acid, glutathione (which helps to stabilize the ascorbic acid), and 10 per cent serum. Heart fibroblasts from the chick, embedded in horse plasma, proliferated two or three times as rapidly in this medium as in the solution of Vogelaar and Erlichman, and remained alive for a much longer period. Baker's medium, with the omission of serum, has been employed with good results by Wilson, Jackson and Brues[273] in a study of glucose utilization, nitrogen metabolism, and lactate production in cultures of embryo chick tissues.

For the long-continued cultivation of monocytes, Baker[8] found it necessary to change the relative proportions of some of the constituents of the solution for fibroblasts and epithelial cells, and to add members of the vitamin B complex. Chicken monocytes continued to proliferate in this medium for eighty days, whereas control cultures in 25 per cent serum diluted with Tyrode's solution did very poorly. The complete formula for the monocyte medium is given in Table III.

In 1939 Baker and Ebeling[13] attempted to devise a fluid that would maintain cells without promoting growth. The mixture finally adopted consisted of Baker's monocyte medium with the addition of tryptophane, sodium glycerophosphate, urea, glycerin, thymus nucleic acid, adrenalin chloride, suprarenal cortex hormone, and pituitary hormones. The presence of all ten of these additional substances was observed to improve the nutritive and maintenance value of the medium. The differences observed when each ingredient was tested separately were, however, too small to constitute definite proof that all were essential. Cultures from a long-continued strain of fibroblasts were maintained in this medium in viable condition and with little or no proliferation for periods varying from forty-three to fifty-six days.

TABLE III

COMPOSITION OF BAKER'S GROWTH-PROMOTING MEDIUM FOR MONOCYTES*

	per 1000 ml.	
Witte's peptone	850.00	mg.
Cysteine hydrochloride	11.25	
Hemin	0.0045	
Thyroxine	0.00113	
Glucose	2000.00	
Glutathione	3.40	
Vitamin C	0.85	
Vitamin A (containing vitamin D)	1000.00	units
Vitamin B_1	0.053	
Vitamin B_2	0.001	
Insulin	0.12	
Serum	150.00	ml.

* This medium also included the salts of Tyrode's solution.

The studies of Carrel, Baker and Ebeling, and Vogelaar and Erlichman demonstrated clearly the important role of proteins and protein degradation products in the nutrition of animal cells in tissue culture; they also emphasized that complex natural substances, such as serum or embryo extract, could not be entirely eliminated from the media. The inclusion of insulin, thyroxin, and other hormones, a noteworthy feature of these media, implies a concept of cell nutrition that has not persisted, since hormones have been absent from all the mixtures that have recently been devised. Whether the omission of these substances is altogether advisable is rendered somewhat doubtful by the recent work of Woolley[275] on the growth factor *strepogenin*. This substance, believed[276] to be a peptide of small molecular weight, has been found[242] to be essential for the growth of several bacterial species, and of certain animal species as well. Strepogenin is isolated[243] from enzymic digests of crystalline proteins, and, significantly, its yield is highest from crystalline insulin. Moreover, Davidson and Waymouth[69] observed certain unidentified growth factors for fibroblasts in the polypeptide and nucleotide fractions of pancreatin. These substances appear to have many of the properties of strepogenin. Thus, the inclusion of hormones in these early media may have supplied a source of active groups, such as strepogenin, which are present as part of the complex protein molecule, and which may exert growth-promoting activity quite independently of their hormonal properties.

It is also of interest to observe that these early workers realized the importance of vitamins in cell nutrition, and that supplements containing all the vitamins then recognized were added to their synthetic media.

A somewhat different approach to the problem of a synthetic medium was taken by Simms,[239] who reported that *serum ultrafiltrate* could be used as a basic medium for tissue cultures. The ultrafiltrate was prepared[240] by dialyzing ox serum under pressure through collodion membranes. The resulting fluid was free from proteins detectable by trichloracetic acid, but contained appreciable amounts of nonprotein nitrogen (0.015 M) as well as other unidentified constituents. According to Simms,[239] the ultrafiltrate contains a substance, the *A factor*, which stimulates the growth of dormant adult tissues *in vitro*, and appears to be essential for the maintenance of living adult cells. He found the A factor to be stable, and apparently an acid of small molecular weight, but was not able to establish its identity.

Serum ultrafiltrate has been recommended[240] for use as a basic fluid medium for the preparation of cultures free from fat deposits, for the study of growth stimulation and inhibition, and for use as a medium for tissue cultures in which viruses are being propagated. For these purposes, serum ultrafiltrate possesses considerable advantages due to the absence of appreciable protein material.

In 1946 Fischer[103] published a monograph summarizing some of the work that he and his collaborators had carried out during the previous twenty years. Considerable attention was given to the problem of cellular nutrition, and especially to the nature of growth-promoting substances and to the nitrogen metabolism of tissue cultures.

Fractionation studies[101] on embryo tissue juice revealed that the growth-promoting activity was associated with labile substances of high molecular weight. These were found to occur in the nucleoprotein portion, and have been grouped by Fischer under the term *embryonin*. Somewhat similar results had previously been obtained by Baker and Carrel,[10] who observed that activity was concentrated largely in the protein fraction that could be precipitated by ethanol or carbon dioxide and that this protein consisted of a mixture of nucleoprotein and glycoprotein. Davidson and Waymouth[67] found that the enzyme mucinase reduced the activity of embryo extract and concluded that muco- or sulfopolysaccharides might be responsible for at least some of the growth-promoting properties. The importance of the nucleoprotein in embryo tissue juice was also confirmed by Davidson and Waymouth.[70] Moreover, activity seemed to be largely bound up in the protein itself, since no correlation was found[262] between the total activity and the relative proportion of the two types of nucleic acid present, although the ribonucleic acid portion seemed somewhat more important. The exact mechanism of action of embryonin on tissue cells remains largely unknown, although Fischer[103] believes that it functions in a catalytic manner. The relationship of the growth-promoting activity of embryo extract to its nucleoprotein content is in accord with the important role assigned to the nucleic acids in the growth and metabolism of tissues.[64]

In experiments with dried embryo tissue juice, Fischer observed that extraction of the lipids considerably increased the activity; this is in agreement with the earlier results of Baker and Carrel,[9] who found that the lipids of serum, chicken brain, chicken liver, egg, and embryonic tissue had an inhibitory action on the growth of fibroblasts. Their toxicity may possibly be attributed to the use of high concentrations of lipid mixtures, since recent work[265] has shown that many bacteria require lipids for growth. The beneficial effects of the lipids may be masked, however, by inhibitory effects due to the presence of free fatty acids in such mixtures.

Dialysis of plasma, serum or embryo extract against Ringer-glucose solution under aseptic conditions[100] resulted in the loss of low molecular weight substances. That such substances were essential for the metabolism of the cells was shown by the fact that tissue cultures rapidly disintegrated in dialyzed media even though embryonin was present. On this basis, Fischer[103] has designated embryonin a growth-promoting factor, and has employed the term *accessory growth factors* for the dialyzable substances of low molecular weight.

On the supposition that dialyzed media would be deficient in free amino acids, Fischer and Astrup[106] then investigated the ability of amino acid mixtures to compensate for the substances lost by dialysis. The best results were obtained with a mixture based on the Bergmann and Niemann[18] analysis of fibrin. This supplement, which contains lysine, arginine, tryptophane, methionine, histidine, glutamic acid, aspartic acid, proline and cystine, considerably prolonged the duration of life of cells in the deficient medium, but did not completely counteract the state of deficiency. The essential amino acid mixture of Rose,[227] containing lysine, valine, leucine, isoleucine, threonine, tryptophane, histidine, phenylalanine, methionine, and arginine, proved considerably less beneficial than that of Bergmann and Niemann. This led Fischer to the important observation that cystine occupied a definite key position and that it could not be replaced by methionine. Further studies,[103] in which extracts of red blood corpuscles or of ox heart were used along with amino acid mixtures, established that both lysine and glutamic acid were indispensable.

All of these results would indicate then, that the amino acid consumption of isolated tissue cells is different from that of the whole organism,[103] and that the concept of essential and non-essential amino acids does not apply to tissue cultures.

Astrup, Fischer and Volkert[5] studied the distribution of substances that could be substituted for the dialyzable constituents of serum or embryo juice, and found that active preparations could be obtained from kidney, heart, yeast, barley malt, and calf embryo muscle. The partial purification of these substances was carried out without significant loss of potency, but further purification was not successful. The addition of small amounts of these products to dialyzed media restored the ability of the media to produce cultures that were normal in growth and appearance.

TABLE IV

COMPOSITION OF FISCHER'S SUPPLEMENTARY MEDIUM, V-605*

	Mg. per 1000 ml.		*Mg. per 1000 ml.*
Glucose	800	Thiamin	3.0
Mannose	100	Riboflavin	0.2
Galactose	100	Pyridoxine	0.3
Inositol	20	Choline	10.0
Adenosinetriphosphate	200	Pantothenic acid	0.07
Fructose diphosphate	100	Biotin	0.007
β-Glycerophosphate	100	*p*-Aminobenzoic acid	1.0
Inosinic acid	30	Ascorbic acid	2.0
l-Lysine	15	Methylnaphthahydroquinone	0.005
l-Histidine	5	Nicotinic acid	0.3
l-Arginine	2	Cozymase	5.0
dl-Valine	14	Glutathione	5.0
l-Leucine	9	Creatine	10.0
dl-Isoleucine	10	Hypoxanthine	100.0
dl-Threonine	12	Sodium succinate	10.0
dl-Phenylalanine	7	Sodium fumarate	10.0
l-Tryptophane	2	Sodium malate	10.0
dl-Methionine	6	Sodium oxaloacetate	10.0
Cystine	5	Glutamine	250.0

* This medium also included dialyzed plasma, dialyzed embryo extract (*embryonin*) and certain inorganic salts.

Fischer and his associates[107] next attempted to devise a mixture of all the substances of biological importance known to be present in animal tissue, and drew up the supplementary medium *V-605*, shown in Table IV. The addition of this mixture to dialyzed media resulted in a rapid growth of fibroblast cultures with cells of normal appearance. In further experiments, it was observed that many of the constituents of this medium could be eliminated without appreciably altering the amount of growth obtained, and a simplified mixture, *V-614*, was devised (Table V).

TABLE V

Composition of Fischer's Supplementary Medium, V-614*

	Mg. per 1000 ml.		*Mg. per 1000 ml.*
Glucose	2000	*dl*-Isoleucine	20
Fructose diphosphate	200	*dl*-Threonine	24
l-Lysine	30	*dl*-Phenylalanine	14
l-Histidine	10	*l*-Tryptophane	4
l-Arginine	4	Cystine	10
dl-Valine	28	Glutathione	10
l-Leucine	18	Glutamine	250

* This medium also included dialyzed plasma, dialyzed embryo extract (*embryonin*) and certain inorganic salts.

The substance found by Fischer to give the greatest stimulation was *glutamine*. Next in importance as a growth stimulant was fructose diphosphate, while the amino acid mixture seemed to be relatively less important. Many other metabolites were tested, including the B vitamins, purines, four-carbon dicarboxylic acids, adenosine triphosphate, cozymase, and trace metals, but no appreciable effects were obtained with any of these substances.

Fischer's findings in respect to both glutamine and fructose diphosphate appear to indicate growth stimulation through the energy reactions of the glycolytic cycle, since both substances are known to be involved in these processes.[186] In this connection it should be noted that earlier studies by Lipmann[171, 172] and by Pomerat and Willmer[216] have furnished indirect evidence that the main source of energy for the growth of tissue cultures is probably not glycolysis. This earlier observation is difficult to reconcile with Fischer's demonstration of growth stimulation by intermediary metabolites of the glycolytic cycle. It may well be, however, that tissue cultures require the *initial* addition of high-energy phosphate compounds to tide them over until such a time as their own cellular enzyme systems are able to supply sufficient energy for the synthesis of protoplasm.

Fischer's observation[107] that no vitamins or coenzymes were required to supplement dialyzed media was interpreted by him to indicate that such substances might be retained in the dialyzed plasma in combination with proteins. Consequently such low molecular weight compounds as glutamine and fructose diphosphate, which were found by Fischer to be growth stimulants, do not necessarily represent all the accessory factors required for cell metabolism, but only those that can be removed from natural media by simple dialysis. It should also be pointed out that Fischer was able to remove all organic phosphorus from his dialysates of embryo juice without interfering with their biological activity, and that such dialysates contained no glutamine. It is probable, therefore, that embryo juice contains other, more complex, accessory growth factors than those identified by Fischer.

In subsequent studies, Fischer[104] attempted to determine which of the amino acids in his mixture, *V-605*, were actually essential. This supplementary medium was altered by the removal of valine, leucine, isoleucine, threonine, and phenylalanine, and by the addition of glutamic acid, aspartic acid, and proline. By withdrawing each of several amino acids in turn, he was able to establish their relative importance for growth and survival. As he had observed previously,[106] the most important single amino acid was found to be *cystine*. In its absence, growth was prevented and the cultures degenerated. Cystine could not be replaced by methionine, but could be replaced by glutathione. A deficiency of glutamic acid was found to retard the growth rate slightly. When glutamic acid was replaced by glutamine, the rate of growth was increased enormously and the appearance of the cells became normal. A marked decrease in growth resulted from deficiencies of arginine, or of histidine and proline, while the absence of tryptophane retarded the growth rate only slightly. From these results, Fischer arranged the amino acids in the order of their decreasing importance as cystine, arginine, tryptophane, glutamine, and lysine. This would appear to indicate that differences exist in the amino acid requirements of the various cell types and that one amino acid mixture will not necessarily suffice for all tissues. Fischer also employed crystalline trypsin and pepsin to prepare digests of pure lactoglobulin and bovine serum albumin. He then devised mixtures of pure amino acids in the proportions found in these proteins, and compared the cell growth that was obtained. Invariably the protein digests caused a much greater growth response than did the amino acid mixtures. This finding is an indication that tissue cells respond quite differently to amino acids and to polypeptides, and that tissue cultures may require an unidentified growth-promoting factor of peptide or polypeptide nature.

Recently White and Lasfargues[264] employed the methods of Fischer[100, 107] to investigate the nutritive value of dialyzed plasma and embryo juice. In confirmation of Fischer's results, they reported that such dialyzed media did not support the growth of frontal bone fibroblasts and brought about a rapid breakdown of the implanted cells. They observed, however, that dilution of the dialyzed embryo juice restored its capacity to support a residual amount of growth, and that the degree of restoration was proportional to the degree of dilution, within the narrow dilution range employed. White and Lasfargues attributed the failure of dialyzed media to support growth to a positive toxic effect rather than to a nutritional deficiency. Although these conclusions were based upon experimental evidence that was extremely meager, they do suggest that dialyzed plasma and embryo juice may not be nutritionally inert and that findings based upon the use of dialyzed media should be interpreted with caution.

The second and more direct approach to the problem of devising a synthetic medium was employed in 1946 by White,[263] who reported a culture fluid consisting entirely of constituents of known composition (Table VI). The cultures were prepared from chick embryo tissues that were grown in roller tubes directly on the surface of the glass (see p. 200). By this method, complex materials, such as plasma, serum and embryo extract, were eliminated, and the effect of various mixtures of chemically known growth substances could be tested directly. Using this synthetic mixture, White reported that he maintained skeletal fibroblasts of eight-day chick embryos in good condition for fifty-eight days, and kept heart muscle beating for forty-four days. Epithelium and nerve cells were grown for shorter periods.

TABLE VI

Composition of White's Medium*

	Mg. per 1000 ml.		*Mg. per 1000 ml.*
dl-Lysine	156	Thiamin	0.10
dl-Methionine	130	Pyridoxine	0.50
dl-Threonine	130	Niacin	0.50
dl-Valine	130	Riboflavin	0.10
l-Arginine	78	Inositol	0.50
l-Histidine	26	Pantothenate	0.10
dl-Isoleucine	104	Biotin	0.10
dl-Phenylalanine	50	Choline	5.00
l-Leucine	156	Folic acid	0.001
dl-Tryptophane	40	Carotene	0.10
Glutathione	1	Vitamin A	0.10
Cysteine hydrochloride	1	Ascorbic acid	0.50

* This medium also included a modified Locke's solution containing iron.

White investigated the influence of amino acids and found that a mixture based on Madden and Whipple's[181] formula for a serum substitute gave best results. This mixture consists of the ten essential amino acids of Rose,[227] but does not contain glutamic acid or cystine, the two amino acids found by Fischer to be so important in cell nutrition.

White also considered the possible importance of trace elements in a synthetic medium, and tested iron, zinc, copper, manganese, cobalt, molybdenum, iodine, fluorine, bromine, silicon and boron, singly and in various combinations. All were tolerated singly, but any considerable group of them suppressed growth. White did, however, incorporate ferric nitrate in the final medium which he reported. Fischer and his colleagues[99] also investigated trace elements and found that, under the conditions of their experiments, iron, copper, manganese, zinc, and cobalt had no beneficial effect. They concluded that their dialyzed media probably contained trace metals in a bound state.

White's medium contains a very extensive vitamin supplement, particularly those members of the B group. But reference should be made to certain omissions, e.g., *p*-aminobenzoic acid, vitamin D, vitamin E, and vitamin K. Moreover, the mixture does not include purines or pyrimidines, and contains no glutamine or fructose diphosphate. Also, in criticism of White's work, Fischer and his colleagues[107] have pointed out that the large amounts of embryo tissue pulp added to the roller tube cultures would provide the cells with a source of unknown growth-promoting substances.

Jacoby and Darke[143] have tested White's synthetic mixture on cultures of fowl macrophages grown directly on glass in Carrel flasks. They observed that White's mixture caused rapid decimation and clumping of cells, and cytoplasmic granulation. But the addition of 10 to 20 per cent serum resulted in good cell survival for periods up to two months.

Morgan, Morton and Parker[194] have also used the direct, building-up method of approach in an effort to develop a completely adequate medium of chemically known constitution. Tissues derived from the leg muscle of eleven-day chick embryos were cultivated directly on the surface of the glass in roller tubes. In contrast to White, however, these investigators reduced the amount of tissue in the roller tubes to a minimum in order to eliminate, as far as possible, the carry-over of nutrient substances from the embryo. All cultures were maintained in a medium consisting of horse serum, chick embryo extract and a modified Tyrode's solution for a period of three to five days, at the end of which time the original medium was replaced by synthetic mixtures. These mixtures were renewed three times a week, and the cultures were maintained until no living cells could be detected. By a gradual building-up procedure, starting from an amino-acid basal solution, these investigators arrived at the complex mixture (No. 199) shown in Table VII. In this medium, the cultures remained in good condition for an average period of four to five weeks, and certain cultures survived for more than twice that time.

TABLE VII

Composition of Morgan, Morton and Parker's Medium No. 199*

	Mg. per 1000 ml.		*Mg. per 1000 ml.*
l-Arginine	70.0	Thiamin	0.010
l-Histidine	20.0	Riboflavin	0.010
l-Lysine	70.0	Pyridoxine	0.025
l-Tyrosine	40.0	Pyridoxal	0.025
dl-Tryptophane	20.0	Niacin	0.025
dl-Phenylalanine	50.0	Niacinamide	0.025
l-Cystine	20.0	Pantothenate	0.01
dl-Methionine	30.0	Biotin	0.01
dl-Serine	50.0	Folic acid	0.01
dl-Threonine	60.0	Choline	0.50
dl-Leucine	120.0	Inositol	0.05
dl-Isoleucine	40.0	*p*-Aminobenzoic acid	0.05
dl-Valine	50.0	Vitamin A	0.10
dl-Glutamic acid	150.0	Calciferol (Vit. D)	0.10
dl-Aspartic acid	60.0	Menadione (Vit. K)	0.01
dl-Alanine	50.0	α-Tocopherol phosphate (Vit. E)	0.01
l-Proline	40.0	Ascorbic acid	0.05
l-Hydroxyproline	10.0	Glutathione	0.05
Glycine	50.0	Cholesterol	0.2
Cysteine	0.1	Tween 80 (oleic acid)	20.0
Adenine	10.0	Sodium acetate	50.0
Guanine	0.3	*l*-Glutamine	100.0
Xanthine	0.3	Adenosine triphosphate	10.0
Hypoxanthine	0.3	Adenylic acid	0.2
Thymine	0.3	Ferric nitrate	0.1
Uracil	0.3	Ribose	0.5
		Desoxyribose	0.5

* This medium also contained a modified Tyrode's solution.

Mixture 199 is characterized by a complete supplement of amino acids and vitamins. It also contains nucleic acid constituents, purines, pyrimidines and pentose sugars, as well as certain intermediary metabolites and accessory growth factors. Glutamine, which was found by Fischer[107] to be of great importance in his supplementary media, is present in this mixture and was observed to have a beneficial effect. The incorporation of Tween 80 is another interesting feature of Mixture 199 and represents a departure from other synthetic media that have been devised. This compound was added in order to supply a water-soluble source of fatty acid (in this case oleic acid) and was also found to be useful as a means of dissolving the fat-soluble vitamins and cholesterol.

Although the medium of Morgan, Morton and Parker represents the most complete mixture, in terms of known growth factors, that has yet been devised, it does not include members of several important groups of intermediary metabolites, and does not contain trace metals, aside from iron and those that may be presumed to occur in the inorganic salt solution employed. It should also be pointed out that the period of preliminary cultivation in serum and embryo extract might perhaps allow the cultures to store nutrient materials that would then be available to the cells during the subsequent maintenance period in synthetic media.

Employing Mixture 199 as a basal solution, these investigators have studied the effect of the supplementary addition of serum and embryo extract. They observed that the addition of serum, from 0.1 to 20.0 per cent, did not significantly improve the growth-promoting qualities of the synthetic mixture. But the addition of embryo extract, at concentrations of 0.25 to 0.50 per cent, proved highly beneficial and resulted in culture growth comparable to that obtained in media comprised of serum and embryo extract. These authors concluded, therefore, that some factor, or factors, present in embryo extract, was still missing from their synthetic medium.

It should be emphasized that in order to be truly synthetic a medium must consist entirely of substances of chemically known composition. In the past, the term "synthetic" has been used somewhat loosely to describe media that, in many instances, contained such poorly defined substances as Witte's peptone or serum. Media containing even very small amounts of complex naturally-occurring materials should be designated as *semi-synthetic*. The term "artificial," which has also been used erroneously to designate culture media, implies that such media are unnatural or counterfeit. And though a completely adequate synthetic medium for animal cells in tissue culture has not been devised at the time of writing, the increasing interest in the problem and the encouraging results obtained during the past few years offer considerable hope that success may soon be achieved.

Chapter IX

COVERSLIP CULTURES

ALTHOUGH the coverslip procedures to be described in this chapter constituted the first tissue-culture techniques and have been replaced almost entirely by the more elaborate flask and roller-tube techniques, they are still useful as a means of preparing short-term cultures for careful microscopic study and as a means of acquiring deftness and precision in handling tissue explants and in developing cell strains. The procedures involved in preparing the tissue fragments, in transplanting the cultures to new media, and in isolating from them homogeneous cell strains are essentially the same for all types of cultures. The coverslip techniques also provide a simple and inexpensive means of preparing living material for classroom demonstrations. But because the ratio of the medium to the mass of tissue in the ordinary coverslip system is so small, and the air volume so limited, the cell colonies have to be subdivided and transferred to fresh coverslips every two or three days in order to prevent the cultures from degenerating.

To make the description more complete, the present discussion will be confined to the procedures involved in the isolation and propagation of common connective tissue cells, or fibroblasts, derived from the skeletal musculature of nine to twelve-day-old chick embryos. Since connective tissue cells were the first cells to be propagated for long periods (that is, for years), the properties displayed by them in cultures are better known than are those of any other cell type.[47] The medium will consist of adult chicken plasma and chick embryo extract diluted with Tyrode's solution or its equivalent. The plasma should be kept chilled until just before use.

Preparation of Hanging-Drop Cultures

The culture room should be clean and free from dust (see p. 40). All unnecessary traffic to and from the room should be avoided both before and after it has been prepared for sterile work. For this reason, everything required should be assembled before the work is started.

MATERIALS

- table cover (black sateen)
- instruments
- pipettes
- glass tubes
- glass plates
- Petri dishes
- depression slides (round and oval)
- coverslips (mica and glass)
- nine to twelve-day-old chick embryo
- adult chicken plasma, on ice
- chick embryo tissue extract
- Tyrode's solution, or its equivalent
- glucosol solution
- vaseline

- small paper labels (gummed)
- rubber bulbs (nonsterile)
- rack for pipettes and instruments (nonsterile)
- paraffin bath (nonsterile)

PROCEDURE

The supplies are placed on one of the auxiliary tables in the culture room until the sterile cover has been spread on the culture table. The wrappings are then removed from the glassware and other equipment, and everything is arranged in somewhat the order indicated in Fig. 36. Three or four large sterile tubes (e.g., 25 × 100 mm.) are opened and placed flat on the table to the right of the operator. These are for the accommodation of instruments (forceps, platinum needle, cataract knife) required in preparing the tissue. The lower section of the pipette rack, which may be sterilized by flaming before the work begins, is reserved for emergency use for instruments for which there is no other protected spot at the moment they must be set down. To the right of the operator's working partner, a large tube is placed for the accommodation of an ungraduated pipette with which to dispense glucosol solution as and when it may be required. A package containing several medium tubes (see p. 51) is unwrapped and the tubes are placed in a Pyrex tray within easy reach of both workers. It is important that their corks should not have worked out during the handling. An unwrapped tube that has lost its cork is always discarded. Two of these tubes are opened, flamed about the neck and set in the pipette rack. Two fine capillary pipettes are now removed from

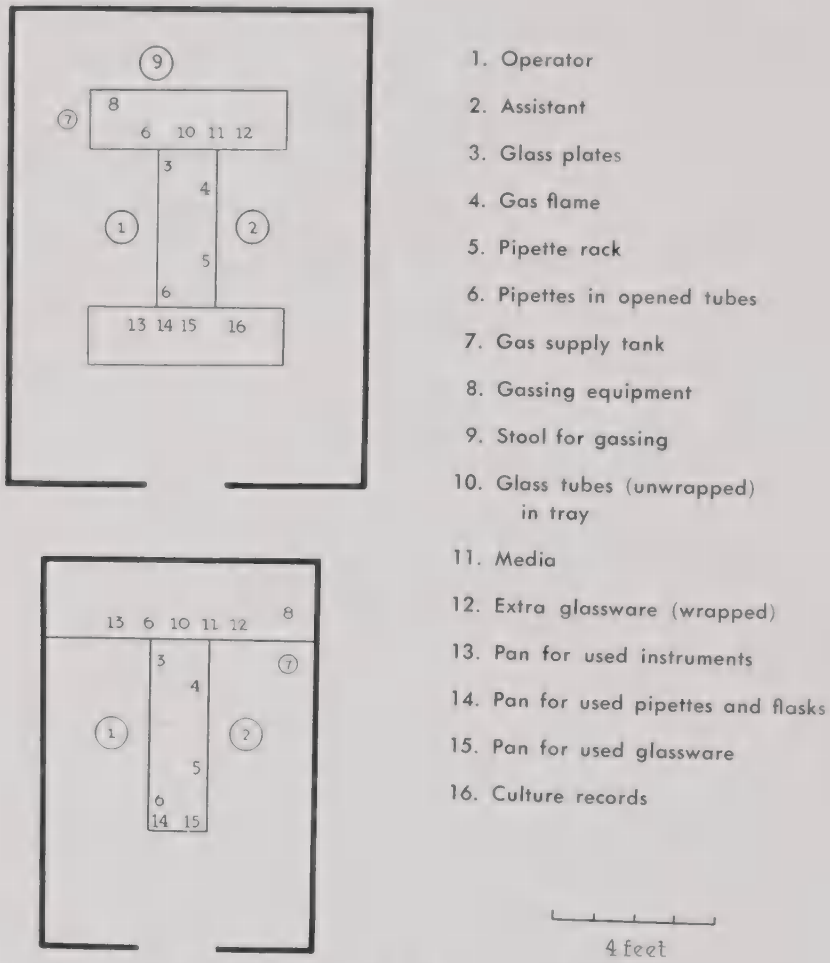

FIG. 36. Convenient arrangement of tables, stools, and equipment for all types of tissue culture work. Each enclosure represents a sterile room of minimum size.

their container, flamed about the top and fitted with rubber bulbs. They are then flamed throughout their entire length and inserted in the tubes that have just been placed in the rack.

The embryo chick supplying the tissue is then removed from its shell (see p. 111) and placed in a sterile Petri dish. One leg of the chick is severed and its muscles are exposed by peeling off the skin. A section of muscle is then removed, transferred to a glass plate, moistened with glucosol and cut into a number of small fragments with a cataract knife. Each fragment should not be more than 1 cubic mm. in size. In the meantime, two or three oval depression slides have been laid on the table and filled with glucosol solution. The moment the slides are removed from their container, they are covered with the top of the dish from which they were taken. This cover is no longer necessary for the protection of the main supply, inasmuch as the slides have been packed, one above the other, depression side down. The tissue fragments are now transferred to one of these prepared slides, and subsequently from one to another in order to free them from blood cells. They are now ready for implantation in the culture medium. Before proceeding, however, the culture table is cleared of all the instruments and glassware no longer required. Used glassware is collected in a separate dish the moment it can be discarded. As each knife is used, it is carefully dried and set aside. It must be remembered that knives, being highly tempered, are never to be flamed. For this reason, they are allowed to remain protected in their box and removed one by one as required.

One of the capillary pipettes is partly filled with plasma, the other with embryo extract. With freshly flamed forceps, four mica coverslips are now placed in a row on the raised section of the table immediately in front of the operator. A single drop of plasma is placed near the center of each coverslip and spread with a cataract knife. During this operation, the coverslip is steadied with a platinum needle held in the other hand. The tissue fragments are then removed from the glucosol bath and deposited in the plasma, one in each drop. Next, a drop of embryo extract is added and the various ingredients are thoroughly mixed with a circular motion of the knife tip (Fig. 37*A*).

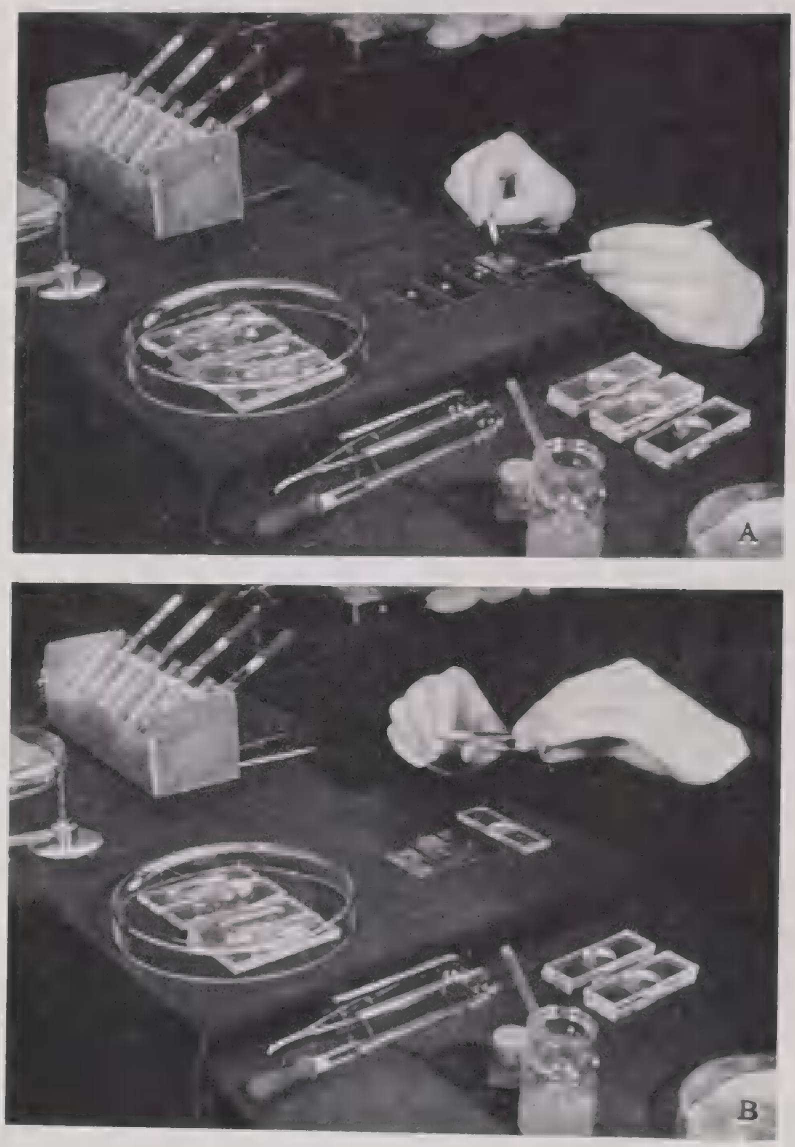

Fig. 37. Preparation of hanging-drop cultures: (*A*) the medium containing the tissue is spread with a circular motion of the knife tip; (*B*) depression slides, bearing a trace of vaseline at each end, are placed down over the coverslips. Eventually, the preparation is inverted and sealed with paraffin.

Care must be taken to avoid scratching the mica more than is necessary. Each drop should be regular in outline and spread to cover an area of approximately equal size throughout the series. It should be neither too small, nor yet so large as to come into contact with the edge of the concavity of the depression slide that is to accommodate it.

Finally, and before coagulation has taken place, the tissue fragments are so oriented as to lie near the center of the medium. In the meantime, several pairs of round depression slides are prepared with vaseline. Since an excess of vaseline is likely to run into the culture upon incubation, only a trace is used. This is applied with a sterile glass rod to three or four slides near the area that will be in contact with the ends of the mica. Each of these slides is now placed face to face with an unprepared slide. The pairs are then separated and oriented, depression side down, over the coverslips (Fig. 37*B*).

By this time, the medium will have coagulated. It will now be possible, therefore, to turn the preparations over, place a small numbered label on one end of each, and set them aside to be paraffined. The vaseline will serve to keep the coverslips in place until another set has been made from the remaining tissue fragments. For the sake of practice, it is suggested that some of the cultures be prepared on glass covers instead of on mica. Finally, when the desired number have been made, they are all sealed with melted paraffin and set away for incubation. The paraffin is applied with a brush around the entire margin of the coverslip. If the preparations are not completely sealed, the cultures will evaporate.

It is extremely simple to prepare temporary slide cultures in which the cells are allowed to live directly on the surface of the coverslip in a fluid medium, without plasma. The tissue fragments are placed in a drop of medium consisting partly of serum. Then, after the coverslip has been covered with a depression slide, in the manner already described, the preparation is sealed from beneath with paraffin and allowed to incubate with the coverslip still on the under side (Fig. 41). If the culture is left undisturbed in this position for twenty-four hours, it may then be inverted for study under the microscope. By this time the cells will have become firmly attached to the glass.

Transplantation of Hanging-Drop Cultures

Perhaps the most exacting technical procedure in tissue culture is that which involves the preparation of subcultures. As already pointed out, hanging-drop preparations have to be transferred to fresh coverslips and new media every few days if active growth and multiplication are to be maintained. In the case of rapidly growing cell colonies, for example, those cultivated in a high concentration of embryo tissue extract, the cultures are transferred more often than when the medium employed is less rich in growth-activating substances. For this reason, the cultures that have just been made should be transferred three times a week.

Before transferring a given set of hanging-drop cultures, one should study them under the microscope in order to determine the particular treatment they are to receive. Cultures to be divided may be so designated by making appropriate inscriptions on the coverslip with a glass-marking pencil. If any portion of a culture is to be discarded, it can be blocked out with pencil. Later, when the coverslip is removed and the culture inverted for cutting, the red or black marking is easily seen and that portion of the culture resting over it may be avoided.

A mica coverslip is nonbreakable, and may easily be pried loose in its entirety from a depression slide. A glass coverslip requires more delicate handling. An old cataract knife, or a razor blade, should be reserved for this purpose. The paraffin is removed as completely as possible from the surface and margin of the coverslip, in order to loosen it more easily and to ensure against an uneven surface. If the surface is not perfectly flat before the coverslip is inverted on a glass plate or a porcelain plaque for cutting (Fig. 38), the pressure of the knife may crack it. When the paraffin has been removed, the sharp point of the knife is inserted, most cautiously, between the cover and the slide, at various points on the margin. The lifting is done with balanced effort from all sides, for every part of the coverslip must be freed from the paraffin and underlying vaseline before any portion of it is raised.

In the matter of preparing the culture transplants, it is first of all essential that the cut surfaces be clean, not ragged. Consequently it is necessary that the knives be extremely sharp and that the culture be cut by a single, quick, forward thrust of the blade, the whole length of which bears upon the coagulum, and with the back of the knife tilted slightly away from the operator. It is also important that the four marginal incisions be made well within the zone of cell outgrowth, at a point where the cell population is fairly dense (Fig. 39). Once a plasma coagulum has been cut, it contracts and becomes thicker. Hence, it cannot be expected that new cells will migrate out from a cut surface consisting solely, or almost entirely, of old plasma. This idea is usually most difficult for the beginner to grasp. He not only tends to overestimate the size of the culture, the margin of which is often extremely difficult to see, but he particularly resents having to leave behind any part of it. After the culture has been entirely severed from the surround-

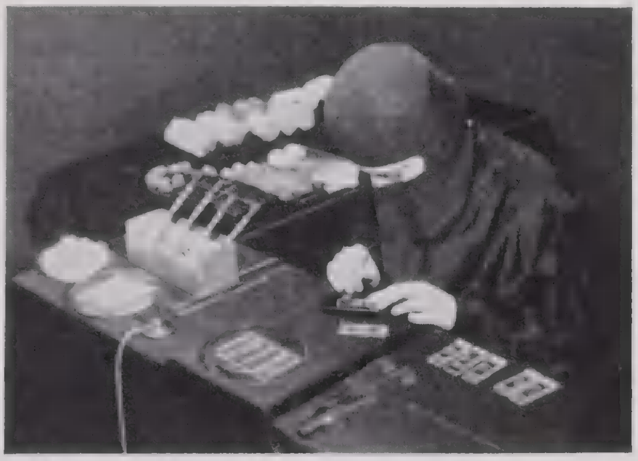

FIG. 38. Hanging-drop cultures being prepared for transfer to new medium. After the paraffin has been removed from the back of the coverslip, the latter is placed for cutting on a glass or porcelain plate.

ing medium, one is in a better position than before to judge the advisability of halving it. And since each incision causes a rapid contraction of both cells and medium, an exact division is more easily approximated if the culture is freed from the surrounding coagulum before the fifth incision is made. Then,

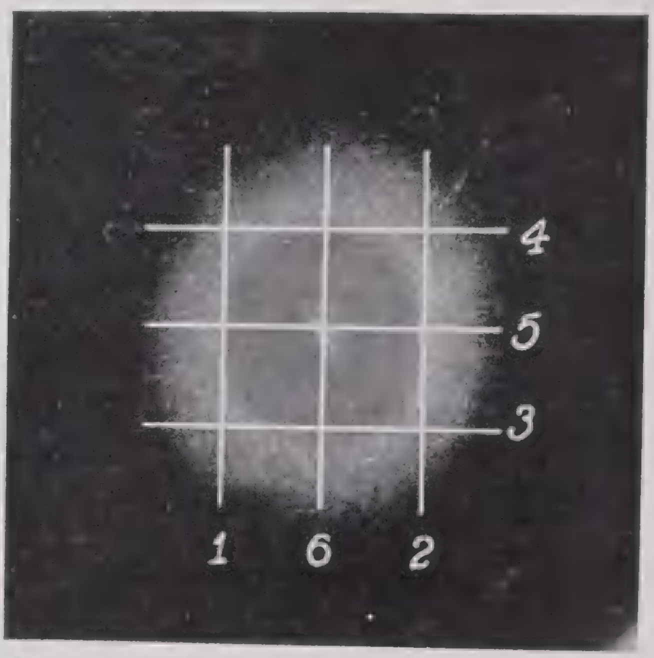

Fig. 39. Incisions made in preparing culture transplants. The thin, marginal areas are eliminated by the first, second, third and fourth incisions. If the culture is to be divided, a fifth incision is made; if four transplants are needed, both a fifth and a sixth are made.

if the halves are large enough to warrant it, they may in turn be divided by a sixth incision. In this way, four subcultures may be obtained. It is important that the beginner should acquire the utmost facility in these operations before attempting to do quantitative work with the more elaborate flask procedures.

It is sometimes difficult to decide whether or not a given culture should be divided at the time of transfer. Size alone is not necessarily the determining factor. If the central area of a culture intended for strain has become unusually thick, the culture should be divided, although it may be smaller than usual. Otherwise, the innermost cells may become necrotic.

When the marginal areas are large enough, however, an even better remedy is to cut away and discard the whole central portion. The marginal cuttings may then be embedded in the new coagulum in close proximity to one another.

It is also important to prevent cell strains from becoming too active. Fibroblasts that are allowed to proliferate too vigorously may digest the fibrin of the coagulum to such an extent as to render their transplantation exceedingly difficult (see p. 173). This condition may usually be anticipated, however, and the danger avoided by reducing the nutritive content of the medium.

Extreme care must also be taken not to mutilate the cultures after they have been cut loose from the medium. Thus, for example, the use of a needle in this connection should never be necessary. A culture fragment is transferred from the old clot to the glucosol bath on the blade of the knife used in cutting it. Once in the bath, it may easily be dislodged from the knife blade, that is, if the fragments are kept well back from the tip of the blade. It should never be scraped off on the edge of the concavity. Similarly, when it is removed from the glucosol solution, the knife (or platinum spatula, if it is being transferred to a flask) is slipped under it and the fragment caught by an upward sweep of the blade. If the culture has become folded around the edge of the knife, one may wash it loose in the plasma by rotating the knife, very quickly, around the long axis, taking care not to crush the cells. In the case of cultures grown on mica coverslips, a thin layer of mica is occasionally cut loose with the tissue and remains adherent to it. The mica should be removed before the culture is embedded in the new medium.

Every effort should be made, of course, to avoid confusing different strains. Each strain should always be transferred separately, although it may sometimes be represented by only a single culture. Under no conditions should the same knife or the same bath be used for two cultures that do not have a common history. These precautions will remove the danger of spreading localized bacterial infections, should they occur, will rule out the possibility of contaminating the cells of one strain with those of another, and will eliminate the possibility of mixing those belonging to the same strain, yet differing in the treatment they have received.

In the matter of *keeping records*, a system should be chosen that will accommodate every type of experiment without being too cumbersome. One that has been found quite satisfactory depends upon the assignment of a number to each culture that is made. When several cultures derived from the same source are grouped in the same experiment, they are given the same serial number with as many subnumbers as there are cultures to designate. Thus, $6781_{1\text{-}8}$ represents eight cultures, the second of which would be accounted for by No. 6871-2. If some or all of these are later transferred to new media, the resulting set receives a higher serial number, i.e., the next number not yet used, and the individual cultures are identified by subnumbers as before, e.g., $6785_{1\text{-}6}$. When such a system is followed, the fate of each individual culture is seen at a glance. If a culture is transferred, its number in the new passage is shown. If it is not transferred, the manner of disposal is indicated. When several cultures derived from a given strain or fragment of tissue are used for separate, unrelated experiments, a new serial number is assigned to each group. The final result makes it possible to trace the history of a given strain or the course of a given experiment with little more effort than is expended in turning the pages.

Printed or mimeographed sheets with wide left-hand margins have been found to be more practicable than bound volumes because extra pages can be inserted to accommodate experiments of more than the average duration, or those involving more than the usual amount of data. The various sheets belonging to current experiments may be kept on a spring-clamp filing board. Finally, at the end of an experiment, the respective sheets are transferred from the current file to a spring binder with hard covers.

RING-SLIDE CULTURES

Although depression slides are convenient to use, they are optically poor and are never suitable for careful study with the light microscope or for photography. A convenient alternative is provided by a simple technique that was originally devised by Gey* and is now used quite extensively. According to this procedure, hanging-drop cultures are prepared on square or oblong No. 1 coverslips and ordinary thin slides, the coverslips and slides being held apart by metal rings of various sizes and thicknesses. The sterile metal ring is dipped in a warm sealing mixture (such as one part vaseline, six parts paraffin), drained for a moment, and placed on the slide. The inverted coverslip bearing the culture is then pressed down over the ring; or the slide and ring are pressed down over the coverslip (Fig. 40). This arrangement gives a preparation with good optical properties.†

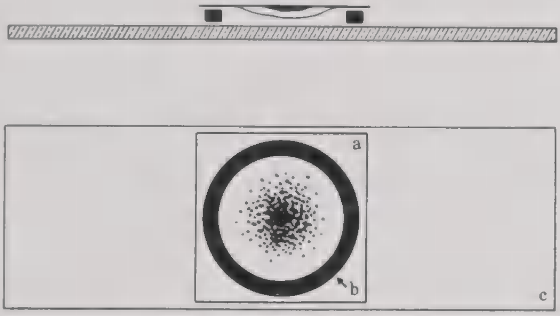

FIG. 40. Diagram representing the procedure followed in the preparation of ring-slide cultures: (*A*) glass coverslip bearing culture on lower surface; (*B*) metal ring that has been dipped in warm sealing mixture; (*C*) slide.

The slides that are used range in size from the small, standard variety (1 × 3 ins.) to ones twice that size (2 × 3 ins.). The outside diameter of the rings that are used is somewhat less than the width of the slides and coverslips that are chosen. Metal rings for the large slides have an inside diameter of 35 mm. and are used with coverslips that are 48 mm. square.

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* Gey, G. O. Personal communication.

† It is of interest to note that Carrel,[39] in 1929, employed a metal ring that was 1 cm. in thickness, with an inside diameter of 5 cm. The top and bottom surfaces of the ring were provided with grooves into which discs of mica were fitted and sealed with paraffin. One of the mica discs accommodated the tissue explants on its inner surface. The wall of the ring was perforated at two opposite points with holes to facilitate the introduction of gas mixtures after the culture had been prepared and the chamber closed.

For more exacting work, the coverslip bearing the culture may be transferred to a thin, perforated slide, as described in the following section.

PERFORATED-SLIDE CULTURES

Excellent optical conditions are provided by a culture system in which glass coverslips are fixed to the two opposing sides of a thin, perforated slide. One of the coverslips bears the culture, and the space between the two coverslips is filled with nutrient fluid, at least in the central area where the culture is located. The coverslip bearing the culture may be attached directly to the perforated slide at the time the culture is prepared, or the coverslip may be attached to a depression slide or to a ring slide (see p. 143) for a day or so and then transferred to the perforated slide a few hours before the culture is to be studied or photographed.

The early perforated slides were of glass and were used by Gey, Carrel, Fischer and others for the preparation of culture material for the microcinema, where the optical requirements are most exacting. More recently, Gey* has devised perforated slides made from stainless steel. The stainless steel slides are 2 × 3 ins. in size, with a circular perforation 1⅜ ins. in diameter. The slide is made from sheet metal approximately 0.029 in. in thickness.

Cultures prepared on depression slides (or ring slides) and intended for subsequent transfer to perforated slides are prepared on coverslips of the appropriate size and attached to the depression slides in the usual manner. For the first twenty-four hours, the cultures are incubated in the lying-drop position, that is, with the tissues resting on the coverslip. On the second day they are inverted, with the culture in the hanging-drop position. This procedure allows the cells to attach themselves to the glass during the early period of incubation, and makes it possible for débris and particles to fall away from the cells, leaving them in better condition for fine cytological study.

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* Gey, G. O. Personal communication.

Eventually, just before the coverslip bearing the culture is attached to a perforated slide, a plain glass coverslip is attached to the other surface of the perforated slide. To accomplish this, the perforated slide is placed in a Petri dish, warmed by flaming, and a few drops of warm sealing mixture (such as one part vaseline, six parts paraffin) are deposited around the opening. The sterile coverslip is then dropped on the slide. If a sufficient quantity of sealing mixture has been used, and if heat is applied, the sealing mixture will spread evenly to make a complete seal between the coverslip and the slide. As soon as the sealing mixture has congealed, any excess is wiped off with gauze, the slide is turned over, and the required amount of fluid medium (about ten drops) is placed on the inner surface of the coverslip. The coverslip bearing the culture is then sealed in place over the perforated slide; and if a couple of spots of vaseline are applied to the slide beforehand, the coverslip will remain in position until the sealing mixture is applied.

If the culture is to be examined or photographed with an ordinary microscope, the sealing mixture may be applied with a brush to the outer margin of the coverslip. But if an inverted microscope is to be used, the sealing mixture for the coverslip bearing the culture should also be applied between the coverslip and the slide, to prevent the preparation from sticking to the microscope stage. The fluid medium that is added should be sufficient to fill the space between the two opposing coverslips, but only in the vicinity of the culture. It should not come into contact with the edge of the perforation.

The fluid medium may consist of 25 to 40 per cent horse serum or human placental-cord serum, Tyrode's solution or its equivalent, and 10 to 20 per cent embryo tissue extract. If a coagulum is required, two drops of plasma or fibrinogen may be mixed with three drops of the fluid medium. For careful cytological work, the medium should be rendered free of particles either by filtering or by centrifugation.

Fluid cultures and cultures containing plasma are handled in much the same manner provided they are started on depression or ring slides and are attached later to perforated slides. But when perforated slide cultures are prepared directly, without starting them on depression slides, the manner in which they are handled depends upon the nature of the medium. When the medium contains plasma, the coverslip bearing the culture is left flat on the table until the medium has coagulated. It is then sealed in place over a perforated slide in the manner already described. When a fluid medium is used, the excess medium required to fill the space between the coverslips is added to the coverslip bearing the culture before the perforated slide (and its attached coverslip) is placed down over it. Wooden racks are used for cultures that are incubated in the inverted position, as lying drops (Fig. 41).

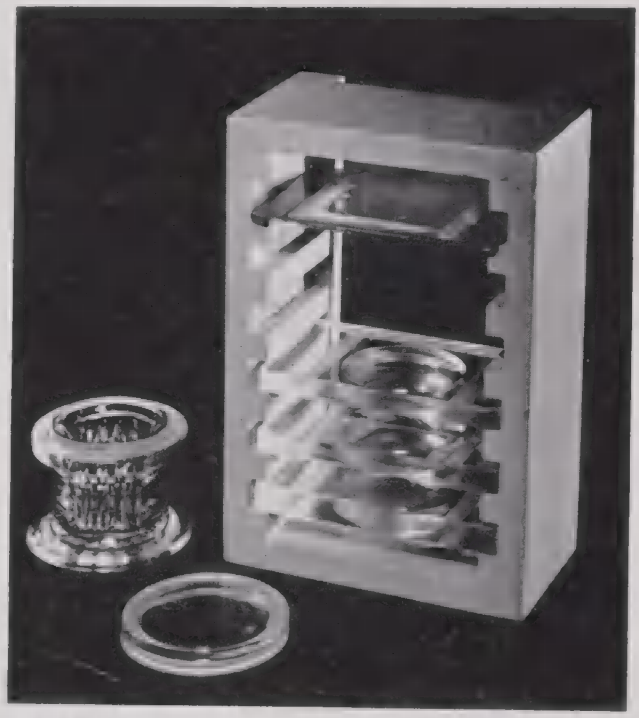

FIG. 41. Wooden rack for the accommodation of perforated-slide and double-coverslip cultures; also, a small Coplin jar (Columbia staining dish) with vertical grooves to hold four coverslips during washing or staining.

Many perforated slide cultures remain in good condition for well over a week, provided the original explants are exceedingly small. It is possible, however, to open the cultures after three or four days in order to replenish the medium. If the sealing mixture has been properly prepared, it may easily be pealed off from the slide while it is still at incubator temperature; when cool, it must be chipped away, with danger of breakage.

Double-Coverslip Cultures

One of the main disadvantages of the ordinary hanging-drop system is that it fails to provide for a change of medium without disturbing the cells. And because the volume of medium in the hanging-drop preparation is so small, it requires changing much more frequently than it would if the volume were larger. In an effort to overcome these difficulties, Maximow[184] devised a double-coverslip type of preparation in which use is made of a large depression slide, 75×45 mm., with a spherical concavity 36 mm. in diameter and 5 mm. deep. The culture is prepared on a small round or square coverslip that is kept in the center of a large mica square by means of a drop of water. The mica square is large enough to cover the depression in the slide and is sealed in place with paraffin. The main advantage of this type of culture rests in the ease with which the inner coverslip, which remains sterile on both sides, may be manipulated in order to wash the culture or change the fluid medium.

To wash the cultures, the coverslip bearing the tissue is dropped into a Columbia staining dish (provided with vertical grooves for the accommodation of four coverslips) containing Tyrode's solution or its equivalent, and subsequently from one staining dish to another, if it is desired to prolong the washing. After the culture has been washed, the coverslip is attached, as before, to fresh mica, a few drops of new medium are added and the mica is sealed to a fresh slide. Ordinarily, the cultures are placed for incubation in a lying-drop position, with the depression slide inverted. For this purpose a suitable rack must be provided (Fig. 41).

Chapter X

FLASK TECHNIQUES FOR PLASMA CULTURES

WHILE the coverslip techniques are useful as a means of studying those properties of living cells constantly manifested *in vitro*, they fail to provide for the long-continued study of a single colony maintained either in the same medium or in one that may be changed at will without disturbing the cells. It is also impossible, with coverslip cultures, to make comparative physiological experiments that are quantitatively accurate. In dealing with so small an amount of medium, it is difficult to measure it accurately and to know how it changes. In an effort to overcome these difficulties, the original flask procedures were introduced by Carrel.[35] During the past twenty-five years, these techniques have been constantly improved and modified to satisfy an almost endless variety of special conditions. Yet the underlying purpose has remained the same throughout, namely, to establish a relatively stable system in which both the effect of the medium on the cells and also the effect of the cells on the medium can be studied simultaneously over long periods. In the present chapter special attention will be given to the cultivation of tissues in a plasma coagulum. In subsequent chapters a description will be given of the procedures followed in the cultivation of tissues in fluid media.

Briefly, the flask techniques for the cultivation of tissues in plasma depend on the preparation of a two-phase system within the flask, one being a permeable, semi-solid plasma or fibrinogen (see p. 170) coagulum in which the tissues are embedded, the other, a fluid phase that may be introduced and removed at will, according to the nature of the experiment. The frequency with which flask cultures are transferred depends on the nature of the tissue, the purpose of the experiment, and the rapidity with which the cells are allowed to multiply. Tissue cells multiply most actively in media containing embryo tissue extract or other growth stimulating substances; in serum alone, they proliferate very slowly. The lower the rate of cell multiplication, the easier it becomes to maintain the cultures in good condition without transferring them. Some years ago, fragments of breast muscle from a twelve-day-old chick were kept alive in single flasks for an entire year without being transferred[209] (Fig. 5). The nutrient materials were supplied by frequent applications of chicken serum diluted with Tyrode's solution. More recently, cultures of the E-0771 mouse mammary carcinoma were kept alive in their original flasks, without transplantation, for six months (Fig. 6).

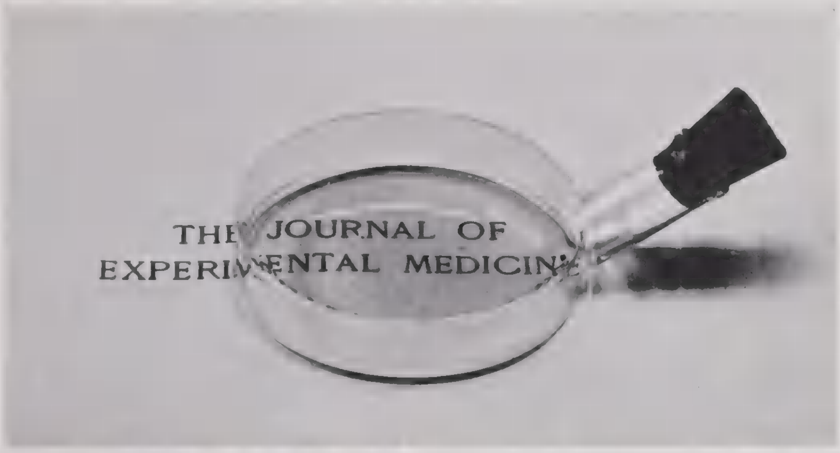

FIG. 42. Carrel D-5 culture flask. Note its excellent optical properties.

Most tissue culture workers have noted that the coagulum of long-continued plasma cultures tends to become more and more opaque the longer the cultures are carried with regular renewals of the nutrient fluid. Hanks[125, 126] has made a considerable study of this phenomenon and is of the opinion that the coagulum adsorbs certain inorganic constituents, particularly calcium phosphate complexes, from the fluid phase of the medium and that, because of this, the fluid bathing the cells of plasma cultures soon comes to have a composition quite different from that intended. Hanks also believes that this process is one of the factors that causes plasma to become growth-inhibitory during prolonged maintenance as a culture substrate. If this is true, the salt mixtures used on plasma, fibrinogen, and possibly also on cellophane cultures, should probably have a quite different composition from those used on cultures maintained directly on glass.

Preparation of Plasma Cultures

It is suggested that the beginner should acquaint himself with the use of D-3.5 flasks before attempting to work with the relatively fragile, thin-walled microflasks. The D-3.5 flasks are of modest proportions and particularly sturdy. Hence, they are especially desirable for general purposes. The only advantage to be gained by the use of microflasks is that the cells may be studied and photographed with oil immersion lenses. But surprisingly good photographs can be taken through the D-3.5 and the larger D-5 flasks (see Figs. 1, 4, 13 and 49). Since these flasks are of heavier construction, they are better adapted to long-continued experiments involving a great deal of handling.

For convenience in describing the preparation of flask cultures, it will be assumed that the tissue fragments to be used have already been prepared according to the procedures described in Chapter IX. They may have been derived from fresh tissue or from cultures previously carried in hanging drops. Moreover, it will be assumed that the material is of avian origin and is to be cultivated in adult chicken plasma and embryo tissue extract, these being diluted with Tyrode's solution or its equivalent. In later sections, attention will be given to the cultivation of avian and mammalian tissues in other types of medium.

The following additions should be made to the materials listed in Chapter IX (p. 133):

platinum spatulas
Carrel culture flasks, type D-3.5
rubber stoppers for flasks
adhesive tape, for labels

To obtain the best optical conditions, the plasma coagulum should be as thin as the nature of the culture will allow. The original clot for D-3.5 flasks is rarely greater than 1.2 ml., or less than 1.0 ml. It might, for chick embryo tissues, consist of 0.3 ml. of plasma and 0.7 ml. of a mixture containing one part 0.01 per cent phenol red made up in Tyrode's solution or its equivalent (to serve as an indicator of *p*H), three parts embryo tissue extract, and three parts Tyrode's solution. When a number of flasks are to receive identical treatment, it is well to mix as many of the various ingredients as possible beforehand. This will ensure their presence in like concentration throughout the entire series. Thus, if six flasks are to be prepared simultaneously, and each is to receive 0.7 ml. of the diluting mixture, 4.2 ml. will be required. However, a slight excess of the fluid should be made up, to give a total of 4.9 ml. comprised of 0.7 ml. of 0.01 per cent phenol red, 2.1 ml. of embryo tissue extract and 2.1 ml. of Tyrode's solution. If, then, 0.7 ml. of this mixture is added to 0.3 ml. of plasma, the tissue extract is $\frac{3}{10}$, the Tyrode's solution $\frac{4}{10}$, and the phenol red 0.001 per cent of the entire medium.

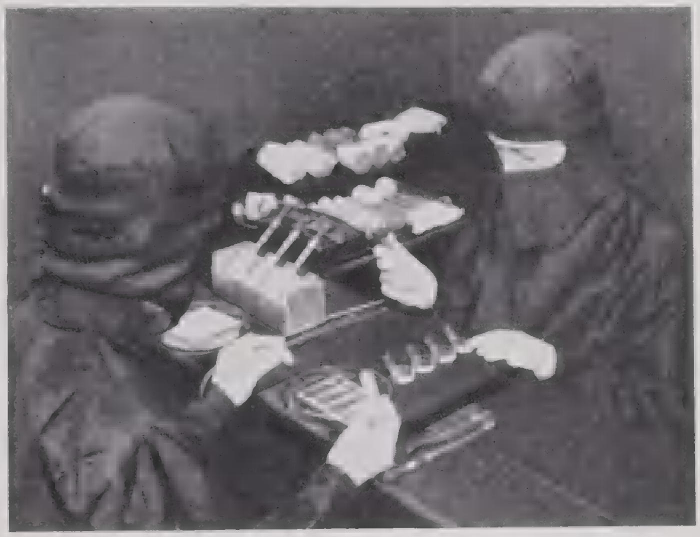

FIG. 43. Preparation of cultures in Carrel D-3.5 flasks.

When everything is in readiness for the actual preparation of the cultures, two straight-tipped platinum spatulas (Fig. 25) are heated to a glow and placed for protection in a large sterile tube to the right of the worker. (Eventually the spatulas are used alternately, so that no time is lost in waiting for one of them to cool.) Four or six flasks are next placed in a row across the table, necks to the right (Fig. 43). The cotton plugs are removed and discarded, and the neck of each flask is carefully flamed by passing an inverted burner over the entire series. With a clean, graduated pipette, 0.3 ml. of plasma is measured into each flask; each is then picked up and shaken in order to spread the plasma evenly over the bottom. The flasks are again flamed about the neck. Into the first is now put 0.7 ml. of the diluting fluid. This is thoroughly mixed with the plasma by gently whirling the flask a few times. To retard coagulation the various media are kept on ice until the moment of use. But as soon as the diluting fluid has been mixed with the plasma, no time should be lost in transferring the tissue from the glucosol solution in the depression slide. The fragment is transferred and oriented near the center of the flask with one of the platinum spatulas. This spatula is then thoroughly flamed and returned to its protecting tube. Or the tissue fragments may be placed in the flask with a plain, ungraduated pipette (one that is neither too fine nor too coarse), provided the operator is able to transfer the tissue fragments with a minimum of fluid. If just enough, but not too much, suction is applied to the pipette, the fragments can be held at the extreme tip and can be dislodged without introducing too much extraneous fluid.

Each of the remaining cultures is prepared in precisely the same manner. After the last one has been finished, all the flasks receive a final flaming before being stoppered. Each rubber stopper is picked up with freshly flamed forceps in such a way that its top may be grasped by the operator and twisted firmly into place. If the flasks are carefully flamed before the sterile stopper is introduced, any amount of moisture that may eventually collect around the inner surface of the stopper will not cause the cultures to become contaminated. When moisture is present care must be taken not to contaminate the material upon opening the flask. The stopper is loosened little by little and all moisture is carefully dried away by constant flaming. This procedure prevents moisture that has come in contact with the edge of the opening from flowing back into the medium. In the author's laboratory, no precautions are taken to keep the medium from coming into contact with the stoppers, yet despite this lack of precaution, there are few contaminations.

Adjustment of *p*H with Carbon Dioxide Gas

It is important to maintain the *p*H of the culture medium at a level that is neither too acid nor too alkaline; and when a number of cultures comprise a comparative experiment, it is important to bring them all to the same *p*H in the beginning and each time they are treated. For most tissues the best growth and survival will probably occur between *p*H 7.2 and 7.6. Blood plasma and serum have, in themselves, a weak buffering action that is greatly augmented by the presence of Tyrode's solution or an equivalent salt mixture. But when large quantities of tissue extract or other unstable substances are present, or when the rate of cell multiplication is high, there is a tendency for the medium to become abnormally acid. If an appropriate indicator, such as phenol red, is incorporated in the medium, it is possible to determine the approximate *p*H of the cultures at any moment by comparing the culture flasks with a set of standard flasks. These standards are made up with phosphate buffers and contain the same indicator in the same concentration as the culture flasks (see p. 85). Phenol red, used in a concentration of 0.001 to 0.005 per cent, is not toxic.

Many years ago, Carrel devised a simple technique for adjusting the *p*H of the medium and keeping it relatively constant throughout the entire course of an experiment. On the day the cultures are made, and as often as necessary thereafter, they are treated with a gas mixture comprised of oxygen, carbon dioxide and nitrogen, the relative proportions of each constituent depending upon the nature of the tissue, the composition of the medium and the purpose of the experiment. The gas mixtures serve a double purpose: First, they supply the cultures with additional oxygen; secondly, they furnish the quantity of carbon dioxide necessary to establish the desired *p*H.

The three gases, oxygen, carbon dioxide and nitrogen, or any combination of them, may be obtained commercially. If it is desired to mix the gases in the laboratory, the mixtures may be made up in 100-pound lots in cylinders that have been emptied with a vacuum pump. By means of a two or a three-way valve fitting, with a 100-pound pressure gauge attached to one of the valve outlets, each gas is fed separately from the needle valve of the supply cylinder into the mixture cylinder until the required pressure (percentage) for that particular gas has been reached. Thus, a mixture including 21 per cent oxygen and 8 per cent carbon dioxide is made up by introducing twenty-one pounds of oxygen, then eight pounds of carbon dioxide, and finally seventy-one pounds of nitrogen. When more oxygen or carbon dioxide is required, the percentage of nitrogen is diminished in like proportion.

Eventually, the gas mixture is bubbled through a saturation flask containing 1 per cent copper sulphate, then passed through a 1 in. "N" Berkefeld filter, to render the mixture sterile, and introduced into the culture flask by means of a sterile glass pipette. The gas is allowed to flow into the flask very gently, at the rate of about 250 ml. per minute; the free end of the pipette is kept well above the surface of the medium. The sterile filter is replaced daily; the glass pipettes are changed as often as there is doubt of their sterility, certainly after several cultures have been treated.

Cultures prepared as described in the present chapter (see p. 151) are likely to be quite alkaline when first made. Before being placed in the incubator, they are gassed with a mixture containing 8 per cent carbon dioxide and 21 per cent oxygen. The flasks are opened one at a time and treated separately. The gas mixture is fed in by means of a sterile pipette for a period of fifteen to thirty seconds, and immediately afterwards the flask is closed with a rubber stopper. After ten or fifteen minutes, the gas will have diffused throughout the medium and the pH may be checked by comparing it with the standard series of buffered flasks referred to on page 85. If the culture is still too alkaline, it is reopened and gassed for a longer period.

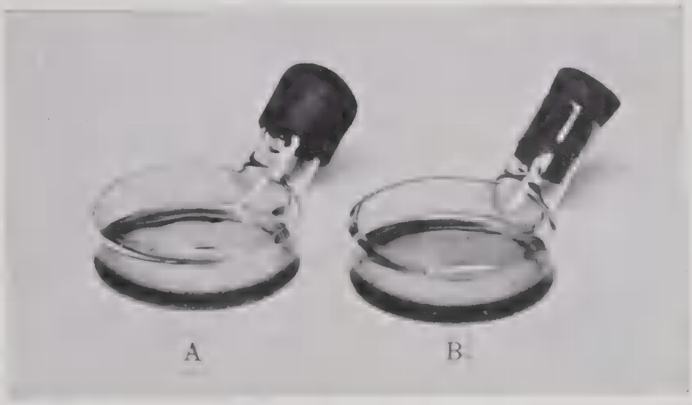

FIG. 44. Carrel culture flasks are sometimes (*A*) closed temporarily with rubber caps in order that excess carbon dioxide may be allowed to escape by diffusion; otherwise (*B*), they are closed with rubber stoppers.

When the culture medium becomes too acid to be adjusted by the displacement of the free carbon dioxide with the usual mixture, or one with less carbon dioxide, the stoppers are replaced with rubber caps (Fig. 44) and the cultures set in the incubator until the excess carbon dioxide has diffused through to the outer air.

With a little practice in testing the effect of different gas mixtures on tissues cultivated for varying lengths of time in different types of medium, the investigator will be able to make the various adjustments accurately and efficiently with little recourse to trial and error.

Lacking all other means, one may adjust the pH of the cultures by treating them with exhaled air. As a rule, this contains about 4 per cent of carbon dioxide. The exhaled air is blown into them through a sterile pipette, the large end of which is plugged with cotton to serve as a filter. If the medium becomes too acid the stoppers are replaced with rubber caps

(Fig. 44*A*) and the cultures are set in the incubator until the excess carbon dioxide has passed off by diffusion.

SUBSEQUENT TREATMENT OF PLASMA CULTURES

After the cultures have been incubated for twenty-four hours, they are "patched" by superimposing a second coagulum upon the first. Patching compensates for any digestion of fibrin that has taken place or that may be expected to occur upon further incubation (see p. 173). It is especially recommended for very active cultures, for example, those cultivated in a medium containing large amounts of tissue extract. If necessary, it may be done on the day the cultures are made, but only after the first coagulum has become quite solid.

Cultures are usually patched with the same medium in the same amount as was used in the original clot. The diluting materials are added first and spread over the entire coagulum before the plasma is introduced. Once the plasma is added, it is thoroughly mixed with the other materials. Before the cultures are returned to the incubator, the *p*H is adjusted by gassing. On the following (the third) day, the cultures receive an additional supply of nutrient materials in the form of a supernatant fluid. For cultures that are prepared from embryo chick tissues and are to be carried as strain, the fluid may consist of 0.3 ml. Tyrode's solution, 0.3 ml. chick embryo extract and 0.001 per cent phenol red (that is, somewhat less than 0.1 ml. of a 0.01 per cent solution). After the mixture has been added and fresh stoppers have been inserted, the cultures are gassed and returned to the incubator.

Two days later, the supernatant fluid is aspirated by suction (Fig. 45*A*) and replaced with a fresh one. The suction is applied by means of a syringe needle that is heated to a glow after each flask has been handled. If one of the cultures has become contaminated, the use of a freshly flamed suction needle eliminates the danger of carrying the contaminating organisms from flask to flask. The needles, which are made from platinum alloyed with 10 per cent iridium, have great hardness and do not readily oxidize.

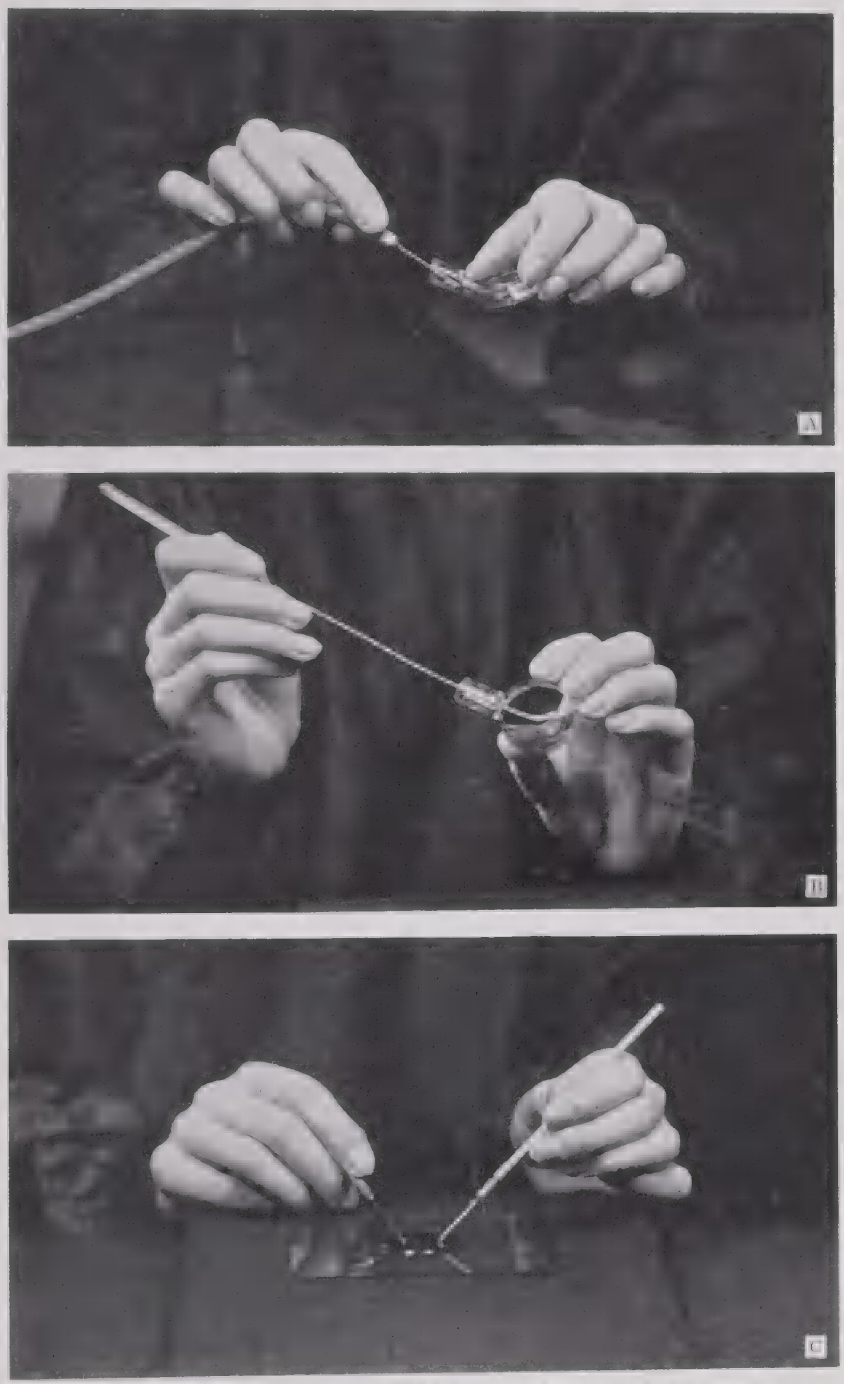

FIG. 45. *A*. Removal of culture fluid from a D-3.5 flask by suction.

B. Plasma culture being freed from the bottom of a D-3.5 flask with the aid of a platinum spatula.

C. Plasma coagulum after it has been removed from the flask (Fig. 45*B*) and deposited on a glass for cutting.

In certain instances, where the plasma of the coagulum has become badly digested, it must be reinforced with a second patch. Under these circumstances another supernatant fluid need not be added until later, if at all. Rapidly growing strain cultures are usually transferred to fresh flasks on the seventh day.

Before patching a culture or a series of cultures for the second time, it is often advisable to reduce the size of the coagulum by inverting the flasks for several hours (or overnight) before removing them from the incubator. The fluid, or a certain definite quantity of it, may then be withdrawn in order to provide more space for air and additional medium. This procedure ensures a quick and easy removal of waste materials.

Individual cultures in a series comprising a quantitative experiment should always be treated in exactly the same manner. If it seems necessary to patch one of them in order to save it, the others should also be patched whether they need it or not. If it is necessary to remove one of the cultures from the incubator for a period of extended study at room temperature, the other cultures belonging to the series should also be removed for the same length of time. For cultures that are designed to test, quantitatively, the effects of particular substances incorporated in the medium, it is important that the substances be present in known amounts and that they be present in the same relative proportions in the original coagulum, in the patch and in the supernatant fluid. Under these conditions, the original coagulum, the patch and the supernatant fluid should each consist of 1 ml. made up as follows: 0.3 ml. plasma, 0.3 ml. chick embryo extract, 0.2 ml. Tyrode's solution, 0.1 ml. phenol red and 0.1 ml. of the substance to be tested. If less than 1.0 ml. is used, it will be more difficult to compute final concentrations. The controls should contain 0.1 ml. of water, if the substance to be tested is in water solution, or an extra 0.1 ml. of Tyrode's solution, if the test substance is in Tyrode's solution. It is also important not to contaminate the tissue fragments with test substances not present in all the flasks or with test substances in greater concentrations than those to be used in cultures still to be prepared from the remaining fragments (see also p. 173).

TRANSPLANTATION OF PLASMA CULTURES

The length of time elapsing before it becomes necessary to transplant plasma culture to fresh flasks and new media depends upon both the nature of the tissue and the rapidity with which the cells have been allowed to multiply. Chick connective tissue cells (fibroblasts) show their greatest activity in embryo tissue extract; but in serum they proliferate very slowly, if at all. The lower the rate of cell multiplication, the easier it becomes to maintain the cultures in good condition without transplanting them. As already indicated, fibroblasts cultivated in serum have been kept alive for a year without being disturbed in any way except by regular changes of the nutrient fluids that bathed them[209] (Fig. 5).

The evening before plasma cultures are to be transplanted to fresh flasks, they are inverted and left in this position (in the incubator) until the time of transfer. This procedure, which serves to drain the fluid from the interstices of the plasma, greatly reduces the thickness of the coagulum and makes it easier to cut the cultures into fragments of equal size.

When a flask culture is transplanted, the entire coagulum is removed from the flask, after which the culture is trimmed and subdivided in the manner already described for coverslip preparations (see p. 139). With a little practice, it will be found possible to remove the discoid clot from the flask in its entirety without tearing it. The flask is held in one hand, and a platinum spatula (Fig. 25) is passed through its neck. With the tip of the spatula, the coagulum is then detached from one side of the flask. The curved elbow of the spatula is used to roll the coagulum back over itself. Care should be taken to manipulate the clot evenly across the entire width of the flask (Fig. 45*B*). As the thin central area containing the cell colony is approached, the utmost caution should be exercised and only the thin, straightened edge of the spatula should be applied. This part of the clot will be found to adhere more firmly to the glass than will the adjacent cell-free areas. Finally, when the coagulum has been entirely loosened from the interior, the neck of the flask is thoroughly flamed and the contents shaken out onto a glass plate for cutting (Fig. 45C).

The transplants prepared for subcultures are placed in depression slides containing glucosol solution (see p. 79) until fresh flasks are made ready to receive them. As before, a separate bath is reserved for each fragment, or group of fragments, taken from any one flask. It is advisable to prepare all of the fragments to be used in a given experiment before introducing any of them into the new flasks.

PROPAGATION OF PURE STRAINS BY SINGLE-CELL ISOLATION

Cell strains are isolated and propagated either as a means of studying the behavior and properties of particular cell types or for use as biological test material. If the strains are isolated as a means of studying particular cell types, it is desirable to initiate them from single, isolated cells. With continued propagation, and under certain conditions of cultivation, the cells of such a strain are almost certain to exhibit wide variations in form and structure; and these variations can be most perplexing unless it is known with certainty that all of the cells comprising the strain were derived from a single cell in the beginning.

Many years ago Fischer[95] observed the multiplication of scattered, isolated Rous sarcoma cells but was unable to derive colonies of normal chick fibroblasts from single cells. He concluded that single, isolated fibroblasts were not able to divide, and advanced the theory[103] that the division of fixed tissue cells depends upon a system of internal exchange between cells through direct protoplasmic connections, or bridges.

In 1935 Moen[193] followed the progressive development of colonies of fibroblasts from single, partially isolated mononuclear cells of the guinea-pig and carried their progeny through repeated subcultures. And while he showed that the presence of other cells in the vicinity enhanced cell division, direct contact with other cells was not necessary for its initiation.

Sanford, Earle and Likely,[233] in 1948, succeeded in establishing the first pure strain (or clone) of tissue cells derived from a single, completely isolated cell (Figs. 1 and 46). Their plan of procedure was based on the theory that the single cell fails to grow when completely isolated because even the best culture medium is so inadequate as to need an extensive modification that can be provided only by the cells themselves. Acting on this concept, these investigators supplied their isolated cells

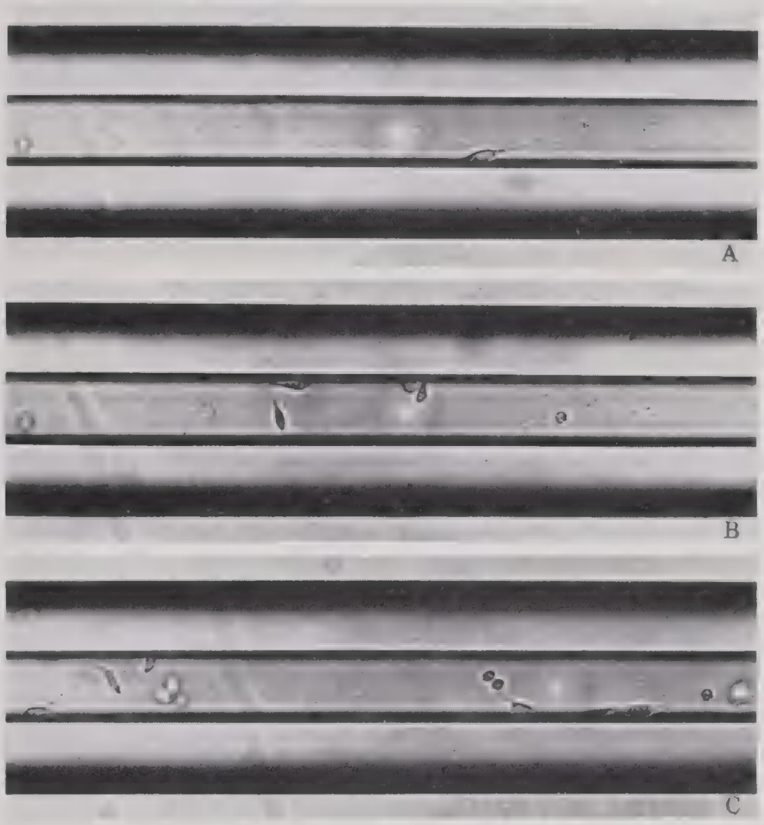

FIG. 46. Origin of Earle's pure strain of mouse cells by single-cell isolation: (*A*) the first cell isolated in a segment of capillary tubing embedded in a Carrel flask in a plasma coagulum with a fluid phase of 'conditioned' medium that was renewed every other day; (*B*) same preparation as in *A*, sixty-five hours later, when six cells were present; (*C*) same preparation forty-eight hours later, when twelve live cells were present. Not all cells are in focus, and one is undergoing division. (K. K. Sanford, W. R. Earle, and G. D. Likely.[233]) × 100.

with cell-free medium that had previously been conditioned by using it as the fluid phase for large plasma cultures of the cell type to be isolated. Thus the medium was modified by the metabolic activity of living cells. The cells were derived from perforated cellophane cultures (see p. 188) belonging to a strain of cells originally derived from normal, subcutaneous connective tissue of an adult C3H mouse. This strain of cells had been treated some years before with 20-methylcholanthrene and had produced sarcomas on injection into C3H mice. Several individual cells were picked by hand from the glass floor of the flask by means of a micropipette inserted through a perforation in the cellophane; and an attempt was made to distribute them along its length. Each micropipette was flushed beforehand with chicken plasma, which formed a thin clot on the capillary wall. After the cells had been introduced, a bubble of air was drawn into the pipette and the end of the capillary was plugged by drawing up a small amount of plasma mixed with fluid medium. The capillary was then broken from the base of the pipette, after which its proximal end and then its distal end were sealed by contact with a molten glass bead. After the sealed capillary tubes had been incubated for fifteen to twenty hours, they were examined under the microscope and well-isolated, healthy, single cells were selected and their exact location was recorded. Then, after the capillaries had been sterilized by immersion in chloroform, they were placed in a sterile Petri dish and broken into 4 to 5-mm. segments, each of which contained a single cell. With forceps, the capillary segments were then inserted into Carrel flasks in 1 ml. of medium consisting of equal parts of plasma and the conditioned culture fluid already mentioned. When the clots had hardened, 1 ml. of conditioned culture fluid was added to the surface of each and the flasks were set away for incubation. The supernatant fluid was renewed every forty-eight hours. Only about 4 per cent of the cells survived for forty-eight hours after planting. From the second to the third day after isolation, the rate of cell cleavage varied from zero to three divisions per cell per twenty-four-hour period. From the third to the fifth day, the rate of cleavage was slightly higher.

It is interesting to note that in all of Earle's preparations no single cells divided in normal, unconditioned medium, and the only single cells to divide were those located near the center of the capillary tube. Later, when the cell density increased, the center of the capillary tube seemed less favorable, for cells in this area became necrotic and died whereas the cells near the open ends of the capillary continued to proliferate. Earle also found that even though small clumps of four to six cells might establish themselves in the capillary tubes in unconditioned medium and cause successive waves of healthy cells to migrate from the ends of the capillary into the outer plasma, the cells could not grow into a healthy colony unless the outer medium contained conditioned fluid.

Cultivation of Special Cell Types

Strains that are used as biological test material, that is, in testing the stimulating or inhibiting qualities of special ingredients added to the culture medium, should be reasonably homogeneous, but it is not essential that they be initiated by single-cell isolation. Over the years, innumerable strains of mixed-cell origin have been used to advantage in many laboratories. In 1912 Carrel[34] succeeded in isolating from the heart of an embryo chick a strain of fibroblast-like spindle cells (Fig. 2) that continued to live and multiply for thirty-four years. Also, Fischer carried the Rous chicken sarcoma in cultures for three and one-half years[97] and the Ehrlich adenocarcinoma of the mouse for twelve years.[108] These and other cell strains have provided an abundance of uniform test material for physiological studies of every sort and description; as a result, a great deal has been learned concerning the nature of these cells and their specific requirements.

After a strain of spindle cells from heart or skeletal muscle has passed through several transfers, it becomes remarkably uniform, that is, if cell multiplication is kept at a maximum and if the dense, central areas of the culture are laid open by cutting each time the culture is transplanted. Under these conditions sister halves, or quarters, of the same parent culture can be relied upon to give an almost identical response to the same environmental conditions. Certain cell types, present in the beginning, are left behind and disappear either because the medium is not suited to them or because they are overgrown by more active cell types undergoing rapid, continuous proliferation. Other cell forms that are capable of reversible changes, or modulations (Bloom[24]), probably assume a character that is indistinguishable from the majority of the cells comprising the cultures. In any event, spindle-cell cultures carried through several passages of rapid proliferation become progressively more and more homogeneous in both structure and function.

In the author's laboratory, where a constant succession of chick fibroblast (spindle-cell) strains are used in testing a wide variety of substances employed in synthetic media, the strains are rarely carried for more than twenty passages (weeks). After fourteen or fifteen passages, the rate of multiplication decreases for a time and for several passages thereafter the individual cultures are rarely large enough at the time of transfer to enable one to divide them into four equal transplants, which is the procedure ordinarily followed. As a result, fresh strains are started every few weeks; and there are always on hand strains of different ages all of which are used to advantage from the time of isolation. During the first few passages (weeks), the cultures are used in preliminary experiments designed to test the toxic levels of substances not previously studied or else they are used in experiments that are not of a quantitative nature. Then, as the strains become progressively older and more uniform in behavior, they are used for more critical experiments. Barring accidents, it is possible, of course, to carry most cell strains indefinitely. But every cell strain that has been isolated has, for one reason or another, shown the same fluctuations in rate of proliferation. Hence, the most practical means of providing a continuous supply of vigorous test material is to develop a succession of strains at regular intervals. This procedure also renders it easier to discard a strain when it is no longer needed, for it has been observed that test strains that are carried for more than twenty weeks gradually become endowed with sentimental values that are not in keeping with their actual usefulness.

In developing a strain of fibroblasts by the technique of gradual purification, the greater part of the original tissue explant may often be removed and discarded at the first transfer by cutting out the entire colony (well within the sparsely populated peripheral zone), halving it, and dissecting out the dense central area. If the marginal pieces are too small for individual transplants, they are re-embedded in the new medium, side by side and in direct contact with each other. Or else, marginal cuttings from various sister fragments may be re-embedded together. Thereafter, the cultures may be multiplied at the time of transfer by the simple process of dividing each colony into two or four equal portions. By following the usual techniques, it is not impossible to derive a strain of fibroblasts from almost any part of the embryo or adult form of any bird or mammal, including the human. In practice, however, the most vigorous strains of fibroblasts usually are obtained from the breast and skeletal musculature of ten to twelve-day-old developing chick embryos. Ordinarily, these are transferred to fresh flasks and new media every seven days. A set of ten or twelve cultures will enable one to make an elaborate quantitative experiment each week and still have enough cultures left over at each transfer for the continuation of the strain.

In the isolation of epithelial strains, the problem is often complicated by the presence of fibroblasts. Under these conditions, it is necessary to separate the fibroblasts from the epithelial elements by mechanical isolation at the time of transfer. By the preparation of a long series of cultures from the fresh explants, and (if necessary) by repeated subdivision, it will be possible sooner or later to cut from one or more of the cultures a sheet of epithelium that has become isolated from the neighboring fibroblasts. Pure cultures of epithelium from epithelial tumors and from certain organs (such as late rat embryo kidney and chick intestine and lung) may often be obtained by macerating the tissues before they are placed in the culture flasks. Under these conditions innumerable microscopic colonies eventually appear and increase in size until they are large enough to transplant. Epithelial cultures can be left in their original flasks or tubes, without transplantation, for long periods, provided the fluid phase of the medium is changed at least twice a week. Under these conditions, cultures of mouse adenocarcinoma have been kept in a healthy, active state for six months (Fig. 6).*

Blood Leucocytes in Plasma Medium

Blood leucocytes derived from whole blood provide excellent test objects for biological study. They are derived from the adult organism, they may be cultivated with or without a plasma coagulum, and they soon become indistinguishable from the so-called tissue macrophages. In 1914 Awrorow and Timofejewskij[6] reported the successful cultivation of blood cells from several cases of myeloid leukemia. Eight years later Carrel and Ebeling[46] described a simple procedure for the cultivation of leucocytes from the buffy coat of centrifuged blood. Since that time, the techniques have been greatly improved and used extensively not only in the preparation of cultures for biological test material but also in the study of some of the peculiar properties of blood cells and blood serum. When fragments of buffy coat are used as test material, that is, to measure the stimulating or inhibiting effects of substances introduced into the medium, they are usually cultivated in a plasma coagulum. Under these conditions, the leucocytes exhibit the very convenient property of invading the medium at a uniform rate from all sides of the explanted fragment. Consequently, within an hour after the cultures have been prepared, the original fragments will be surrounded by a dense corona of cells, and colonies supplied with highly stimulating substances will be

* Parker, R. C. Unpublished experiments.

much larger than the controls. These differences become more pronounced during the next few days.

As soon as the blood has been drawn, the tubes are packed in ice and centrifuged as in the preparation of plasma (see p. 96). After centrifugation, the supernatant plasma is pipetted off and stored in plain glass tubes to coagulate. The serum is then extracted according to one of the techniques described in Chapter VI. In the meantime, however, the leucocytes are prepared.

In withdrawing the plasma from each tube of centrifuged blood, a fine capillary pipette is used to remove the last traces of plasma, as well as any froth that may have formed. Then, with a second pipette, a drop of chick embryo tissue extract is deposited, very carefully, on the surface of the leucocyte layer (buffy coat) and spread by rotating the tube. These operations must be carried out very gently so as not to disturb the leucocytes and cause them to mix with the underlying red cells. After the tissue extract has been added, the blood tubes are set aside at room temperature, for a half hour or so, to await the coagulation of the leucocyte layer. The time required for coagulation will depend upon the peculiarities of the donor, the amount of extract added, and the temperature of the room. With a little experience, the investigator will be able to judge from the general appearance of the layers approximately when they are ready for use. Otherwise they are tested from time to time by withdrawing the cork and sliding a flat platinum spatula around the periphery of the layer, in order to loosen it from the wall of the tube. If the leucocytes are firm enough the entire layer is folded over the spatula and transferred to a glass plate for cutting, or else placed temporarily in glucosol solution. If by this time, however, the underlying red cells have also coagulated, the leucocytes may still be salvaged by loosening the entire coagulum from the tube and shaking the cylindrical mass out onto a glass plate. A cataract knife, moistened with glucosol, is then used to cut the leucocyte layer away from the adherent red cells. After the discoid layers have been washed several times in the salt mixture, they are placed on a fresh plate and cut into strips and fragments for the preparation of the cultures.

Each fragment should be clean, firm and from 1 to 2 sq. mm. in surface area. If a series of cultures is to be made, the fragments should be of equal size. As the fragments are cut, they are returned to a fresh glucosol bath (in a depression slide); and before the cultures are prepared, the fluid is drawn off and replaced a few times. If several tubes of blood are available, and the first one fails to yield a solid layer, another may prove to be better. It so happens, however, that the blood of certain individuals always gives soft, unstable leucocyte layers from which it is sometimes impossible to obtain clean, solid squares.

Once the leucocyte fragments are ready, the cultures are prepared in the manner described earlier in the chapter (see p. 150). But in an experiment designed to test the effect of substances that may be inhibiting or stimulating, the medium should contain very little, if any, tissue extract. If the cultures are left undisturbed for a few minutes after they have been made, the plasma will coagulate spontaneously and without the addition of any extract whatsoever. Otherwise a single drop may be added.

Where the nutrient materials consist entirely of plasma, or plasma and serum together, they should comprise from one-third to one-half of the total medium. If other nutrient substances are present, or if plasma and serum diluted with Tyrode's solution are being used as a basic control medium for experiments designed to test the effect of other substances, then the amounts may be reduced to one-fourth or one-sixth, according to the particular concentration of plasma required to produce a solid coagulum.

Unless the plasma is of heterologous origin, and the cultures are to be washed before the final medium is added (see p. 170), the coagulum should contain all the ingredients required in the experiment. It is seldom necessary to reinforce the coagulum by patching. On the second or third day, the cultures should receive a fresh supply of nutrient materials in the form of a supernatant fluid. It is always advisable that this fluid consist of the same materials as those contained in the coagulum, in the same relative proportions, serum being used to replace the plasma. In a subsequent section (see p. 180), a description is given of the cultivation of leucocytes in a fluid medium.

Cultivation of Tissues in Heterologous Plasma

In the cultivation of certain tissues, it is often desirable to prepare the supporting structure of the solid phase from the plasma of another species, for any one of the following reasons: (1) The homologous plasma may give a coagulum that is opaque, weak, or uneven in density. (2) The homologous plasma may be difficult to obtain. (3) A particularly valuable sample of blood may coagulate before plasma can be prepared from it, or the plasma itself may coagulate spontaneously before the cultures are made; in either case, the material can be used only in the form of serum. (4) Certain tissues, such as mouse epithelium and rat tumors, may digest homologous fibrin but not the fibrin of another species.[49, 163, 235] In many instances of this sort, the tissues are cultivated in a chicken plasma coagulum containing heterologous serum and other nutrient substances compatible with the tissue. Chicken plasma is particularly well adapted for this purpose; it is easy to obtain in large quantities and provides a firm, transparent coagulum that can be treated almost indefinitely without sacrificing its rigidity.

The chicken plasma coagulum may be prepared from 0.5 ml. of plasma and 0.7 ml. of a mixture consisting of two parts horse serum, two parts Tyrode's solution or its equivalent, and one part chick embryo extract. A day or so later there is placed on the clot, as a supernatant fluid, 0.5 or 1.0 ml. of the same mixture of horse serum, Tyrode's solution and embryo extract. Because horse serum has the convenient property of slowing down or preventing the digestion of the fibrin clot (see p. 173), cultures prepared in this manner do not require frequent patching. It is only necessary to renew the supernatant fluid twice a week. Chicken tissues may also be cultivated in this medium, particularly if it is desired to keep them in the same container without transplanting them for long periods. In fact, it is easily possible to keep mouse, rabbit, human and chicken tissues in a state of active survival in this manner for weeks and months. It matters not whether the tissues are embryonic or adult, normal or malignant. If they are left undisturbed without transplanting them for long periods, they will continue to multiply at a slow rate and to display many interesting properties not observed in short-term cultures multiplying at maximum speed (Figs. 6 and 13). From time to time, it may be necessary to reinforce the coagulum with a second application of plasma.

Some investigators use human placental cord serum (see p. 105) instead of horse serum. Horse serum and human placental cord serum may also be used in the cultivation of heterologous tissues that are cultivated in a fibrinogen clot (see below), directly on glass (see p. 201), or on perforated cellophane (see p. 188).

When the use of heterologous plasma is undesirable beause of the serum it contains, it is best to use a clot prepared from purified fibrinogen and thrombin (see below). But when, for one reason or another, this seems undesirable, the original coagulum, once it is firm enough, may be washed for two hours with two changes of glucosol (see p. 79). If phenol red is to be used in the culture medium, it should also be included in the washing fluid. After the final washing, the cultures receive from 0.5 to 1.0 ml. of the actual feeding mixture. If it is not known whether the clots will withstand this treatment without losing their rigidity, appropriate blanks, without tissue, may be prepared for testing.

Cultivation of Tissues in Fibrinogen

Coagulated blood plasma has long been accepted as the most satisfactory support for the growth of tissue cells cultivated *in vitro*. Of all the plasmas that have been studied, chicken plasma is particularly favored as a supporting structure because it provides a firm, transparent coagulum and one that is particularly resistant to the proteolytic action of tissue enzymes. But all plasma has certain disadvantages: It is tedious and time-consuming to prepare; it sometimes coagulates during storage; and its tremendous complexity makes it impossible to duplicate exactly many types of experiments in which it is used. Different samples of plasma vary in their clotting time, in their chemical composition, and in their general effectiveness as a culture medium. These variations are related to the age and health of the donor and to other factors, some of which may still remain obscure. Futhermore, plasma can be the means of transmission of infectious agents, especially viruses. Because of these obvious disadvantages, repeated attempts have been made over the years to find a more suitable supporting structure.

The first successful attempt to utilize fibrinogen as a substitute for whole plasma was reported by Ebeling[82] in 1921. When the fibrinogen, which was prepared from adult chicken plasma by acetic-acid precipitation, was used together with serum and embryo tissue juices, it provided a medium in which fibroblasts grew almost as well as in plasma. The next attempt to develop culture techniques for the use of fibrinogen was reported by Porter and Hawn[218] in 1947. These investigators, taking advantage of the plasma protein fractions prepared by Cohn and his associates,[57] were able to develop a system of cultivation which, because of the uniformity of the materials and the very considerable amounts that could be obtained commercially, could easily be duplicated in other laboratories. It should be pointed out, however, that the fibrinogen samples thus far available are by no means pure. Certain samples contain as much as 40 per cent citrate, with fibrinogen comprising only 70 per cent of the remaining portion.

The fibrinogen* most generally used is that contained in Fraction I of bovine blood plasma prepared according to the method of Cohn and his associates. It is distributed as a dry powder. A 0.5 per cent solution of the fibrinogen in Tyrode's solution or its equivalent is sterilized by Seitz filtration and stored in the refrigerator. It is used at room temperature at *p*H 7.6 to 7.8. If the *p*H of the solution is below 7.5 during coagulation, or if the solutions are used while still cold, the coagulum is likely to be opaque.[91] Hawn and Porter have shown by electron microscope studies that the opacity of the coagulum is related to the size of the fibers that are formed.[133] The thrombin* used for the coagulation of the fibrinogen is that contained in the Fractions II and III of bovine plasma and is available commercially as a sterile, dry powder designed for topical application. The powder is dissolved in Tyrode's solution to give a final concentration of 20 units per ml. It is then distributed in small containers and stored in a dry-ice box or low-temperature refrigerator. One drop of thrombin solution is sufficient to coagulate 1.5 ml. of a nutrient mixture one-third of which consists of fibrinogen.

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* The Armour Laboratories, Chicago, Ill.

Fibrinogen flask cultures may be prepared in any number of ways provided ample time is left for orienting the tissue fragments after the nutrient materials and the thrombin have been added to the fibrinogen solution. For the small (D-3.5) Carrel flasks, the following procedure is suggested for experiments in which each culture is to receive a different combination of ingredients. The fibrinogen is placed in the flask first and is spread to cover the bottom. The combined nutrient materials for each of the flasks are then placed in short, open tubes (e.g., 18 × 100 mm.) held in an inclined rack; and a drop of thrombin is added to each. If four flasks are to be prepared simultaneously, four tubes of nutrient are required, one for each flask. When all is in readiness for the actual preparation of the cultures, the contents of the first tube are mixed and transferred to the corresponding flask by means of a fresh pipette; and another fresh pipette (or a freshly flamed spatula) is then used to transfer to the flask the tissue fragment, or fragments, previously prepared for it. If 0.5 ml. of fibrinogen is placed in the flask, 1.0 ml. of nutrient may be used; and the materials are mixed by a gentle rotation of the flask just before the tissue is added. The nutrient may consist of two parts horse serum, two parts Tyrode's solution or its equivalent and one part chick embryo extract. Or it may consist of any combination of these or other materials appropriate to the experiment.

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* Parke, Davis and Co., Detroit, Mich.

As an alternative procedure, the fibrinogen may be added to the medium tube at the last possible moment before the contents of the tube are transferred to the corresponding flask. Under these conditions the fibrinogen is added to each tube with a pipette that is used only for fibrinogen, and the combined medium is then mixed and transferred to the corresponding flask by means of a fresh pipette. Another fresh pipette (or a freshly flamed spatula) is then used to transport and place the tissues. It must be remembered that a pipette that has been placed in a particular tube of medium must not be used to transport the tissues unless all the cultures receive the same medium or unless the tissue for each culture is in a separate container.

Use of Antitryptic Agents to Prevent Digestion of Coagulum

The rapidity with which actively growing cells digest the fibrin of the coagulum has presented a very considerable problem since the early days of tissue culture. In 1914 Losee and Ebeling,[178] working with human material, attempted to prevent the plasma digestion by the addition of serum, agar, and egg albumin. Although the experiments were unsuccessful it was found that by diluting the plasma the rate of digestion could be retarded sufficiently to permit short term experiments to be carried out. Years later, Carrel[35] reported that he was able to protect fibrin clots from digestion by the addition of a little serum, a small amount of sodium linoleate, or a suspension of egg yolk.

Of the various sera that have been employed to prevent digestion of the plasma coagulum, horse serum has proved to be the most suitable, although it does have the undesirable property of inhibiting somewhat the rate of cell multiplication. In many laboratories, the coagulum is protected by the addition of a second layer of plasma on the day following the preparation of the cultures (see p. 156). Subsequent layers of plasma are then added as they become necessary.

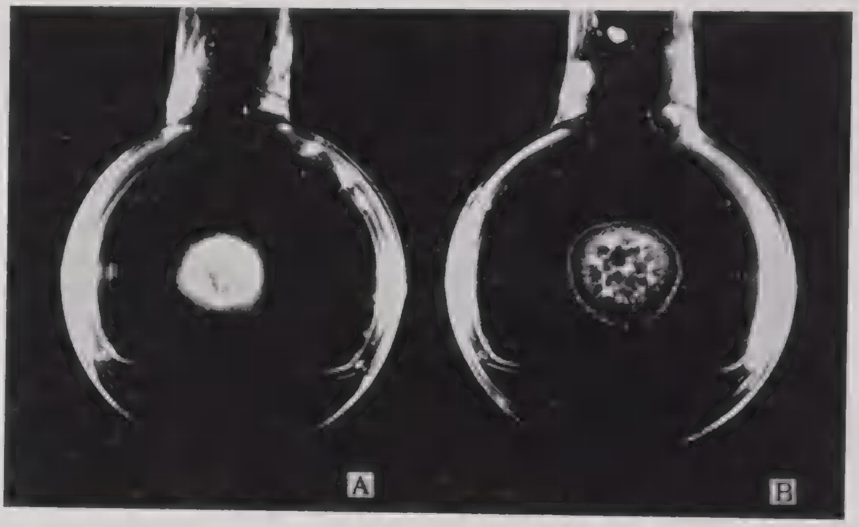

FIG. 47. Eleven-day sister cultures of twelfth passage chick fibroblasts cultivated in a plasma medium containing 0.5 mg. of soybean antitrypsin per ml. (*A*); and in the absence of antitrypsin (*B*). Note the digestion of the coagulum in B. (J. F. Morgan and R. C. Parker.[195]) ×1.

Substances that inhibit the proteolytic action of trypsin have been found in serum and plasma,[118] in egg white,[14] in navy beans and soybeans,[26] and in extracts of pancreas.[152, 147] Because of the great activity of these antitryptic agents, they have been used in tissue culture as a means of preventing clot digestion.

Fischer[105] has described a method for the cultivation of the Rous chicken sarcoma in fowl plasma to which he added crystalline soybean antitrypsin.[151] The antitrypsin, however, was growth inhibiting, an effect which was also observed with normal fibroblast cultures.

Morgan and Parker[195] have studied the action of crude soybean antitrypsin on chick fibroblast cultures maintained in fowl plasma and in fibrinogen-thrombin clots (see p. 170). Antitrypsin levels between 0.25 and 5.0 mg. per ml. completely prevented the digestion of the coagulum (Fig. 47). High levels of the antitryptic agent were found to delay the coagulation of plasma, even in the presence of embryo extract, but this inhibition could be overcome by the addition of a few drops of diluted thrombin. Also, high concentrations of antitrypsin (1.0 to 3.0 mg. per ml.) retarded the rate of cell multiplication. It was observed, however, that by making repeated passages (three to five) through media containing inhibitory levels of antitrypsin, the cultures gradually acquired a tolerance to it. The growth-inhibiting effects of the antitryptic agents may possibly be avoided by the selection of concentrations that are sufficient to prevent digestion but which are below the levels inhibitory to growth. There is also the possibility that antitryptic agents from other sources may be more suitable.

CULTIVATION OF FRESH TISSUE EXPLANTS FOR ASSAY PURPOSES

Cells cultivated *in vitro* are delicate indicators of the presence of cytotoxic substances; they are free from all complicating nervous and circulatory influences and are not subject to the marked differences in local concentration that occur in the intact animal. When a given amount of the material or substance to be tested is placed in a culture, it cannot escape or suffer uncontrolled dilution. Migrating cells must, therefore, wander out into a medium containing in each case the desired concentration of the material under investigation. Earlier in the present chapter (see p. 166), and in the chapter to follow (see p. 180), descriptions are given of the use of blood leucocytes as a source of cells suitable for this purpose. Embryo heart and spleen fragments, short segments of embryo spinal cord, fragments from the larger blood vessels of the embryo, and many other tissues, may also be used. If a suitable antitryptic agent (see p. 173) is incorporated in the medium, these fresh tissues will not digest the fibrin of the coagulum, and the coagulum need not be reinforced as a precautionary measure.

Medawar[187, 188] has devised a hanging-drop test to take advantage of the fact that freshly explanted tissues of a given source and embryonic age possess a characteristic power of resistance to growth inhibition. These tests consist in determining the concentration of a drug that is sufficient and necessary to suppress all outgrowth from a standard explant for twenty-four hours at 37°C. The standard explant is a fragment of the wall of the ventricle of a ten-day-old chick embryo heart, and the standard medium consists of 0.1 ml. of equal parts of fowl plasma, without anticoagulant, and a solution of the drug in balanced salt solution. Serial dilutions of the drug are prepared, at each level of which five or ten explants are incubated as separate cultures. After twenty-four hours it is easy to determine by inspection the concentration that is just high enough to inhibit all outgrowth. In the neighborhood of this "threshold point" concentrations of the drug differing from each other by less than 10 per cent are distinguishable. After forty-eight hours of subjection to the action of the drug, the cultures may be subjected to a further test which consists in washing the excised tissue fragments for one hour in balanced salt solution, at room temperature, after which they are embedded in a plasma and embryo-extract medium devoid of the drug. A later inspection of the subcultures then provides an estimate of the minimum lethal concentration of the reagent used, since it is possible to record that concentration of the drug which, under the conditions of the experiment, the cultures can withstand for forty-eight hours without undergoing irreversible changes. If the drug is relatively nontoxic, the cells are not killed by a concentration *higher* than that necessary to suppress all growth; a more toxic substance may, however, kill the cells at a concentration *lower* than that which allows a certain amount of migration and growth to take place within the first twenty-four hours after explantation.

Pomerat[215] prepares cultures for biological assay from chick embryo spinal cord. The necks of embryos of suitable ages (nine or ten days old, for example) are cut at their base with scissors, and the spinal cord is expelled from the neural canal by compressing the tissue in the middorsal line with two pairs of forceps moved alternately in the direction of the sectioned vertebral column. Thus, both cervical and thoracolumbar segments of cord can be obtained. These are washed in Tyrode's solution, transferred to a plate, and cut with sharp knives into transverse sections varying from ¼ to ½ mm. in length. Heart fragments from the same chick embryos and spleen fragments from older embryos (eighteen to twenty days old) provide other cell types for comparison. Pomerat, employing the hanging-drop method of cultivation (one drop of 50 per cent fowl plasma combined with one drop of 25 per cent chick embryo extract), measures the outgrowth of nerve fibers after twenty-four and forty-eight hours and the outgrowth from heart fragments after seventy-two hours. Four cultures are prepared for each tissue and for each dilution of the test solution, and an effort is made to find: (1) the lowest concentration of the test solution that will prevent all cellular migration from the explanted fragments, (2) the highest concentration that does not inhibit growth and migration, and (3) the minimum concentrations that produce certain graded effects on the cell morphology.

In the author's opinion, these tests should be made either in Carrel flasks or in roller tubes whenever possible. Actually, flasks are more suitable than roller tubes because of the constant diameter and uniform thickness of the coagulum that may be achieved in flask cultures and because of the relative ease with which they may be observed, measured and photographed. Both flask and tube cultures may be carried infinitely longer than hanging-drop cultures before it becomes necessary to terminate the experiment or transplant the tissue fragments to fresh medium. Also, it has been observed that the growth-inhibiting effect of certain toxic materials does not become evident, even in flask cultures, until the third or fourth day, by which time a hanging-drop culture would almost certainly be badly degenerated because of limited food and oxygen, if for no other reason.

For assays made in flasks, the tissues may be embedded in a plasma or purified fibrogen coagulum (see p. 170) containing known amounts of the material to be tested. The material should be incorporated in the balanced salt mixture used to dilute the other ingredients. If an antitryptic agent (see p. 173) is also incorporated in the medium, it should not be necessary to augment the coagulum on the second day in order to ensure against digestion of the fibrin support. Instead, on the second day 1 ml. of fluid medium may be added, and this should contain the test substances in the same concentrations that were used in the original coagulum. On the fourth and sixth day, the fluids should be renewed. On the seventh day, the experiment may be terminated, or, if it seems desirable, the fragments may be transplanted to fresh flasks and new medium that does not contain the test material. In this manner it is possible to determine whether the immediate effects produced by the test material are permanent or whether they are reversible and temporary.

CHAPTER XI

FLASK TECHNIQUES FOR FLUID CULTURES

WHEN tissue cells, either embryo or adult, are cultivated in a plasma or fibrinogen clot, they migrate out into the medium to form a colony, the size, structure and shape of which depend upon the nature of the cells and the manner of cultivation. But all cultures thus grown soon reach a characteristic size that remains almost unchanged regardless of the length of time the colony may be left in its container and the frequency with which the nutrient fluid is changed.[206, 125] The surface area attained by a cell colony depends partially upon the nature of the cells, the character of the medium, and the size of the tissue fragment planted in the medium. The speed with which the colony reaches its maximum size depends upon the rate at which the cells are allowed to multiply. Cells cultivated in high concentrations of embryo tissue juice achieve their maximum colony size in seven or eight days, after which they will begin to degenerate unless they are transplanted. Cells cultivated in plasma and serum, with little or no embryo tissue juice, migrate more slowly, and the culture may not achieve its maximum size for several weeks. Thereafter, cell multiplication and cell death may continue to take place within the colony itself and the culture may remain alive for as long as a year[209] without any apparent change in its surface area.

In striking contrast, however, is the behavior of cells cultivated directly on glass or on perforated cellophane, in a fluid medium. Under these conditions, the cells continue to migrate and to multiply indefinitely, and some form tissue patterns of amazing complexity. A test tube that is rotated continuously may become completely lined with cells, though seeded with but one or two small fragments in the beginning; and the floor of a large culture flask containing a sheet of perforated cellophane may become completely covered with cells that often grow in several layers (Fig. 53*B*). Cell death may be localized and not easily discernible, or the cells of a whole area may degenerate simultaneously and be replaced, eventually, by a fresh migration of cells that move in from neighboring areas.

In the present chapter, attention will be given to techniques for the cultivation of blood leucocytes in fluid media, techniques for the cultivation of tissues on perforated cellophane, and techniques providing a continuous circulation of fluid media. Later sections will be devoted to techniques for the cultivation of tissues in roller tubes, with and without plasma (Chap. XII), and to techniques for the cultivation of tissue fragments in suspension (Chap. XIII). Reference was made to the preparation of coverslip cultures with fluid media in Chapter IX.

Blood Leucocytes in Fluid Media

As mentioned in the last chapter, Carrel and Ebeling[46] in 1922 described a simple procedure for the cultivation of large mononuclear leucocytes derived from the buffy coat of centrifuged blood. When fragments of buffy coat are cultivated in plasma, they provide a culture system that is particularly useful as a means of testing, quantitatively, the inhibiting or stimulating qualities of materials added to the culture medium (see p. 166). When fragments of buffy coat are cultivated in a fluid medium, without plasma, they provide a system in which one may study the special properties of blood cells and blood serum.[48, 42] If the cultures are fed at frequent intervals with 20 to 50 per cent blood serum in Tyrode's solution or its equivalent, they may be kept in an active state for several weeks.

These large mononuclear leucocytes, or monocytes, are extremely sensitive to certain conditions existing in the medium. Their various sensitivities are expressed not only in their rate of migration and multiplication, but also in their morphological appearance. A monocyte readily modifies its size, shape, nucleus, neutral red vesicles, fat granules, mitochondria, number of

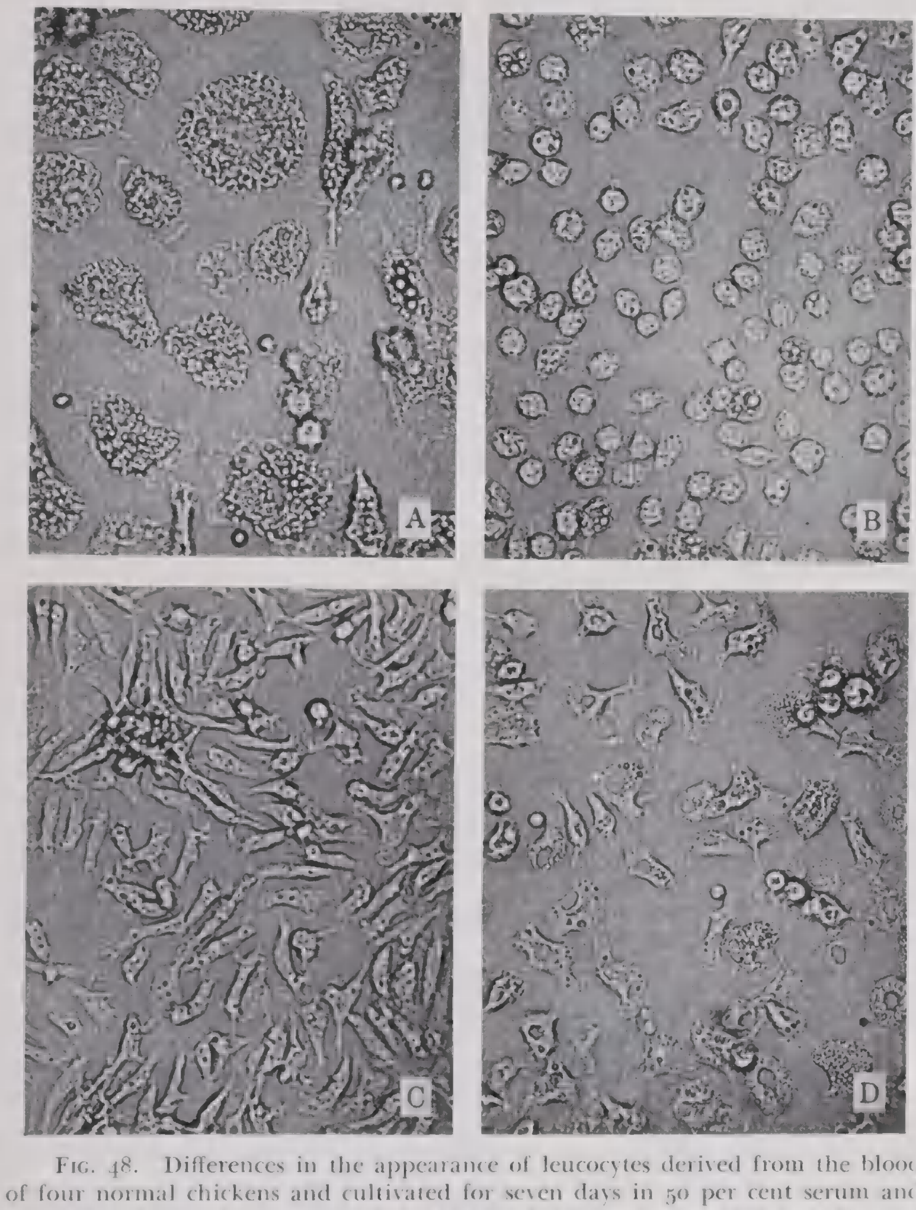

Fig. 48. Differences in the appearance of leucocytes derived from the blood of four normal chickens and cultivated for seven days in 50 per cent serum and Tyrode's solution. In each instance, the leucocytes were cultivated in their own (autologous) serum. (A. Carrel, unpublished data.) ×230.

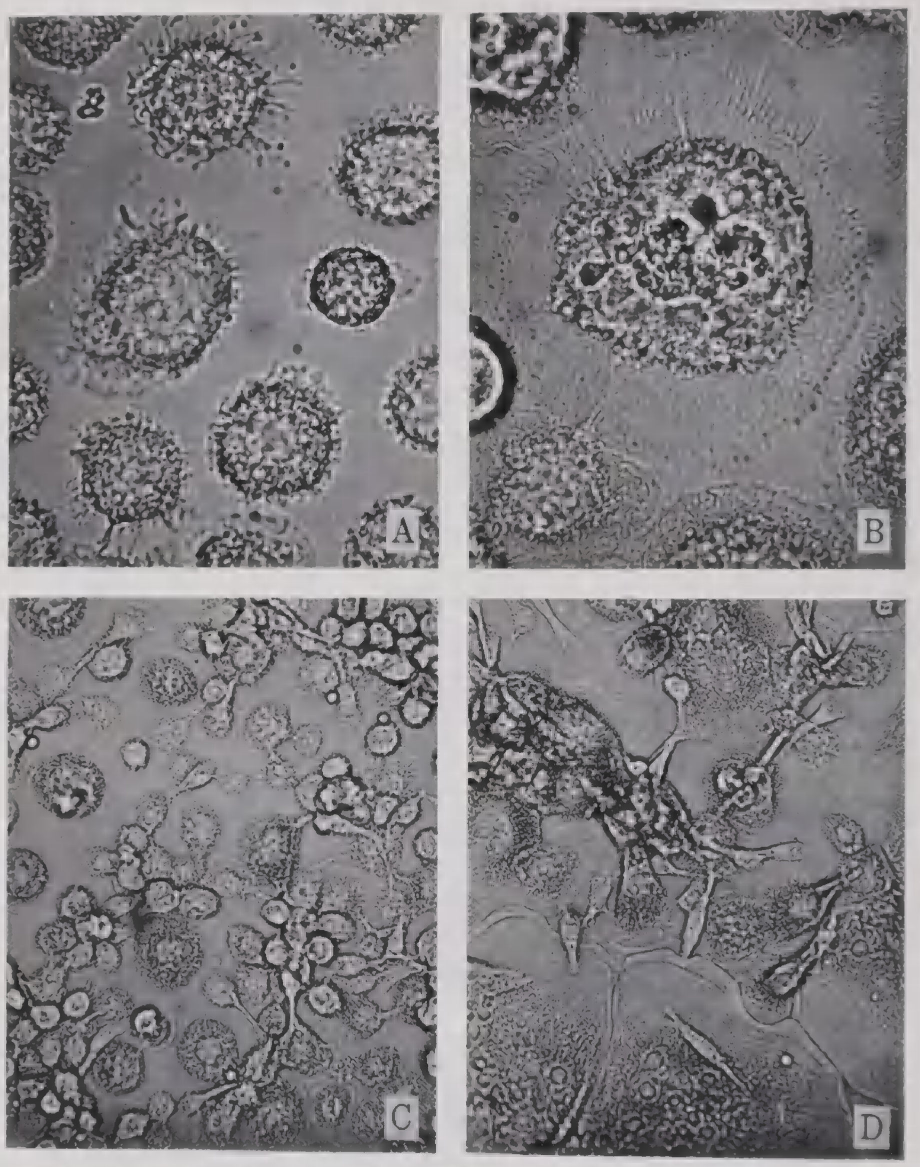

FIG. 49. Human leucocytes derived from the blood of four individuals and cultivated for seven days in 50 per cent serum and Tyrode's solution. In each instance, the leucocytes were cultivated in their own (autologous) serum. (A. Carrel, unpublished experiments.) *A* and *B*, ×620; *C* and *D*, ×230.

nuclei, undulating membrane and mode of association with other cells. All of these characteristics may undergo several variations. Hence, the number of different combinations of these variations is extremely large. In fact, each monocyte or colony of monocytes can be said to have its own individual aspect that distinguishes it from all others (Figs. 48 and 49). Each seems to constitute a portrait which reflects its own innate qualities and also the response of these qualities to the immediate environment (Figs. 50 and 51). As such, then, they provide an excellent source of biological test material. When cultivated in fluid media containing, for example, synthetic nutritive materials, toxic substances and drugs, or in natural media altered by the methods of physics and chemistry, it is possible to study not only the major changes affecting the ability of the cells to survive and multiply but also the lesser changes determining their size and shape and the nature of their cytoplasmic inclusions.

Carrel[42] has further shown that monocytes from different sources tend to take on the same appearance in a given serum. It seldom happens, however, that the blood serum of two normal individuals of the same breed gives an identical appearance to two sister cultures of the same cells. This is not difficult to understand when it is realized that serum contains substances derived from all parts of the body and must therefore reflect some of the actual conditions that make for individuality. It has also been found that when leucocytes from one individual are cultivated in the serum of a second, they assume the appearance of leucocytes from the second individual grown in their own serum.

In other words, the appearance of leucocytes grown in a particular serum is the expression of some peculiar quality of that serum. Carrel was able to demonstrate this very simply by cultivating the leucocytes of a normal and of a diseased animal, each in its own serum and also in serum from the other animal. The results he obtained are illustrated in Fig. 50. When leucocytes from chickens inoculated with Rous sarcoma were cultivated in their own serum, they became round and coarse and

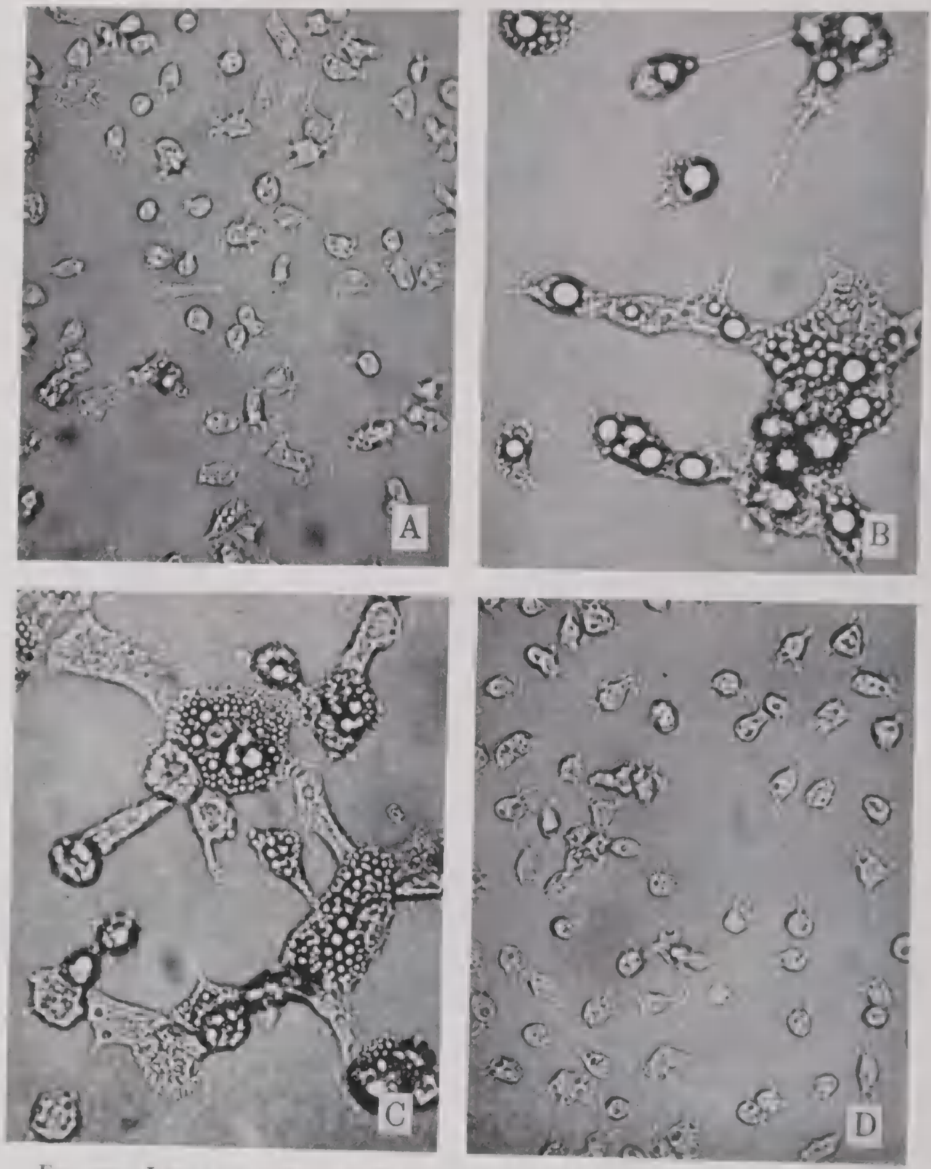

Fig. 50. Leucocytes derived from a normal chicken and from one infected with Rous sarcoma, each cell type cultivated for seven days in 50 per cent serum obtained from both the normal and the pathological chicken. (A. Carrel, unpublished data.) × 230.

A. Normal leucocytes in normal serum.
B. Normal leucocytes in pathological serum.
C. Pathological leucocytes in pathological serum.
D. Pathological leucocytes in normal serum.

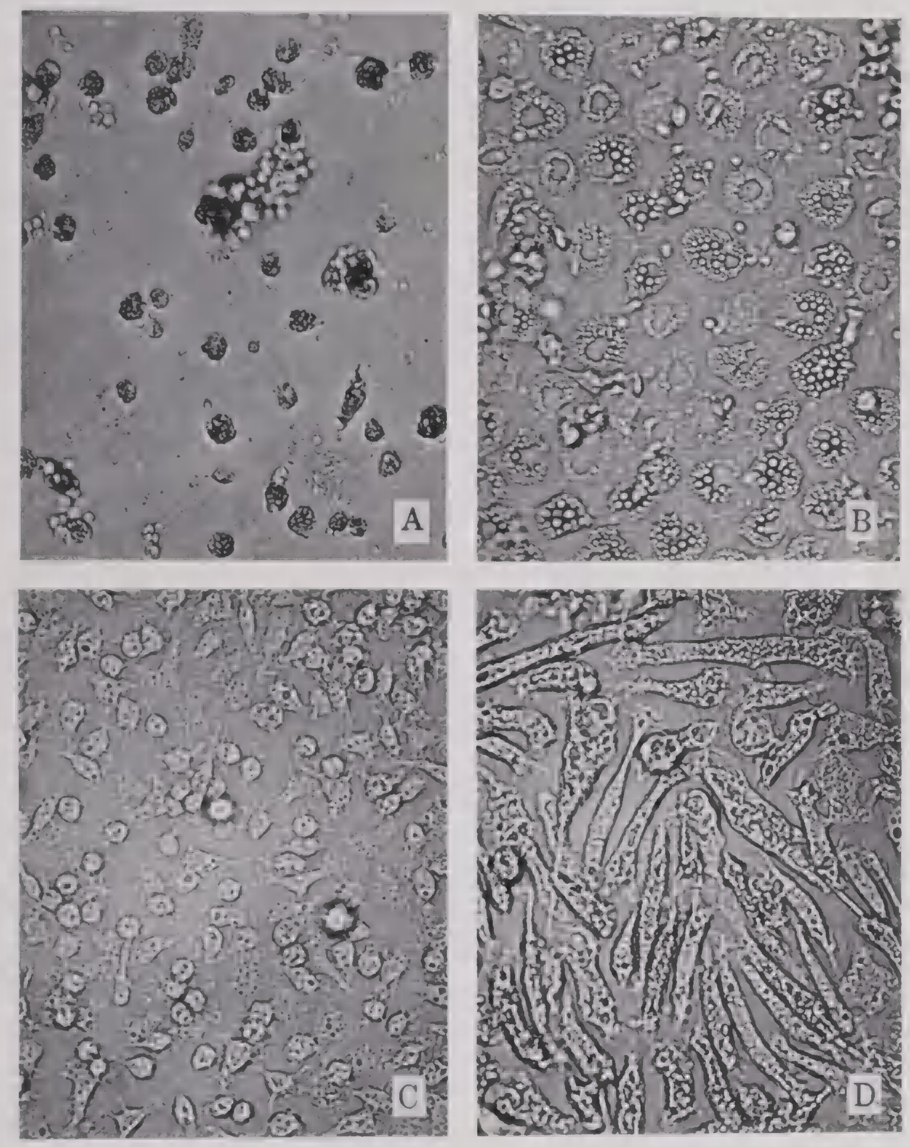

FIG. 51. *A* and *B*. Chicken leucocytes cultivated for ten days in 50 per cent rabbit serum (*A*); and in another sample of the same serum heated for one hour at 55° C. (*B*). (R. C. Parker, unpublished experiments.) × 230.

C and *D*. Chicken leucocytes cultivated for seven days in 50 per cent chicken serum and Tyrode's solution (*C*); and for the same length of time in the same medium to which was added 2.5 mg. per cent thyroxin (*D*). (A. Carrel, unpublished experiments.) × 230.

showed a tendency to agglutinate. When they were placed in the serum of a normal chicken, they lost their pathological appearance and resembled the leucocytes of the normal chicken grown in their own serum. Conversely, the cells of the normal chicken soon came to resemble those of the sarcomatous chicken when they were cultivated in the pathological serum. Also, Carrel, Ebeling and Rhoads* studied the monocytes of patients with Hodgkin's disease, microcytic anemia, and pernicious anemia. When the leucocytes of the patient and those of an appropriate control individual were cultivated each in their own serum and in the serum of the other individual, striking differences were found that confirmed their earlier conclusions, namely, that blood serum impresses upon leucocytes the physiological and pathological peculiarities of the individual providing the serum. Several years later, similar investigations were undertaken by Draper and his associates[75] as a means of studying leucocyte variations among individuals of four disease groups: poliomyelitis, peptic ulcer, coronary disease and gallbladder disease. The techniques for the cultivation of leucocytes in fluid media will now be presented in some detail.

The fragments of buffy coat are prepared in the manner described on page 167. When the fragments are cultivated in a fluid medium, however, the entire medium is placed in the flasks before the leucocyte fragments are introduced. The basic medium may consist of serum diluted with equal parts, or less, of Tyrode's solution containing phenol red. If other ingredients are added, they are introduced in place of a certain amount of Tyrode's solution, unless the medium is completely synthetic and the serum is omitted. The amount of medium depends upon the size of the flasks; microflasks and D-3.5 flasks both require 1 ml.

Where many different substances, or different concentrations of the same substance, are to be tested in a single experiment, it is well to prepare a detailed schedule in advance, with everything ready and at hand in the proper concentrations before the cotton plugs are removed from the flasks. If the experiment is large, as many as eight flasks may be opened at once. Then, without losing any time, the various ingredients are added and mixed together by shaking the flasks. Finally, the leucocyte fragments are introduced by means of freshly flamed spatulas and oriented near the center of the flask. Unfortunately, however, some fragments may not remain there and will have to be reoriented after the flasks have been stoppered, and perhaps still again when the *p*H has been adjusted by gassing (see p. 153). After the flasks have been placed in the incubator, they should be weighted down to prevent tipping, and left quite undisturbed in their original position for three days. By this time the leucocytes will have spread over the larger part if not the entire bottom surface of the flask; they will have become so well attached to the flasks that no amount of jarring, or even washing, will dislodge them. The original fragments will often have lost their identity.

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* Carrel, A., Ebeling, A. H., and Rhoads, C. P. Personal communication.

On the third day the flasks are opened, the medium changed and the *p*H readjusted by gassing. If the medium is renewed twice the cultures should remain in excellent condition for ten days. After this time the cell population may have become so dense and the medium so acid that the latter will have to be changed every two days in order to keep the cells from degenerating. As a rule, however, the experimental results may be read on the seventh day. By this time the cells will have come to express very completely the particular conditions set up in the experiment.

Jacoby, Medawar and Willmer[144] have used macrophage cultures to advantage in testing the toxicity of these cells to various drugs. The cultures were used for experiments after not less than eight days' cultivation in Carrel flasks in a basic medium consisting of 20 to 40 per cent serum and Tyrode's solution. In normal cultures renewal of the medium was followed by a latent period of from twenty to twenty-four hours, after which there occurred a major wave of mitotic activity that reached a peak between the thirtieth and the fortieth hour. Less regularly the major wave was followed by a minor wave with a peak between the fiftieth and the sixtieth hour, after which the cells gradually returned to quiescence.[112] This entire period represented a single growth cycle, the end of which was taken as zero time for testing purposes. At this zero time (about the hundredth hour), a fresh medium containing the drugs to be tested was placed on the cultures and left in contact with the cells for another whole growth cycle, during which time the *mitotic index* was measured by means of serial photography.[268]

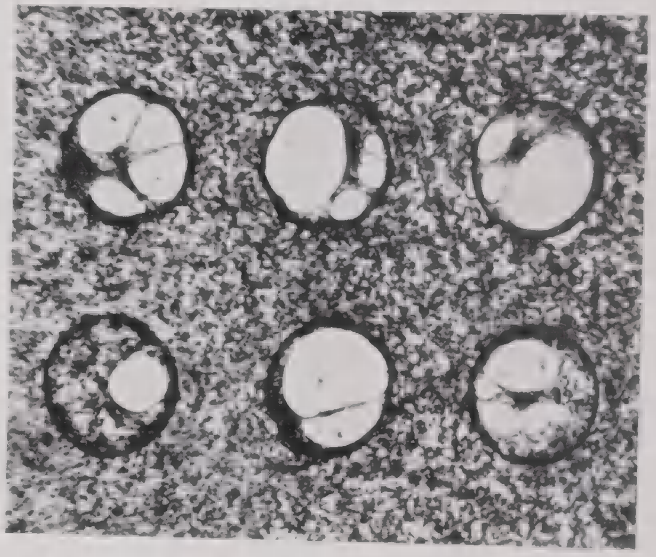

FIG. 52. Chick embryo cells cultivated for sixty-five days on perforated cellophane[87] in a fluid medium that contained 40 per cent chicken serum for the first fifteen days and 40 per cent horse serum thereafter. × 17.

PERFORATED CELLOPHANE CULTURES

Although the plasma coagulum provides the best support thus far obtained for the propagation of tissues outside the body, it has many obvious disadvantages, the main one being its chemical complexity and the fact that it cannot be used in studies having to do with the basic food requirements of tissue cells. At one time and another many chemically inert substitutes for plasma have been investigated, but none of them have proved entirely satisfactory. And though embryonic cells can be grown directly on the surface of the glass (Fig. 57), this method is not so suitable for older tissues. Recently, however, Evans and

Earle[87] have devised an ingenious method for the use of perforated cellophane as a supporting structure. Cellophane had been tried many times before, but it had never been perforated; and it was this additional refinement in the technique that was responsible for its success.

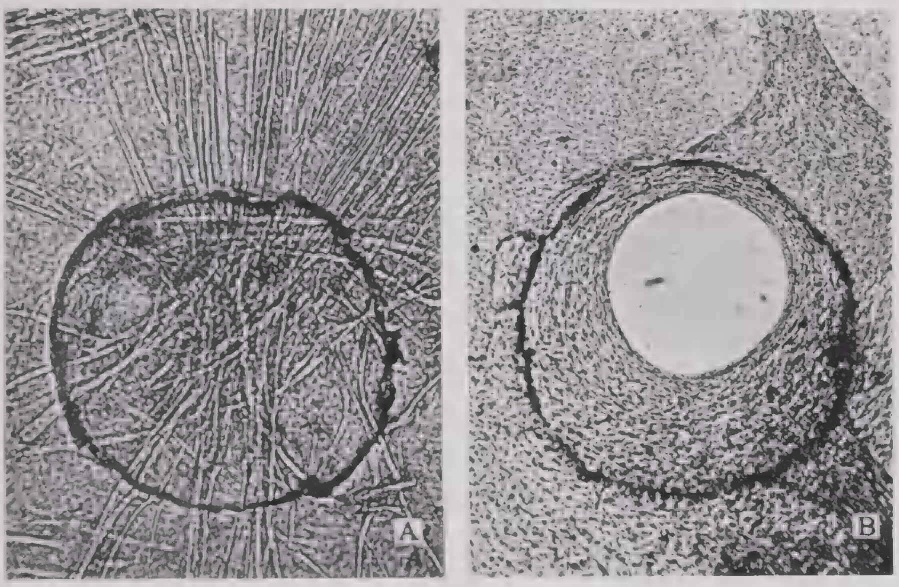

FIG. 53. *A*. Second passage chick embryo cells cultivated for thirty-eight days on perforated cellophane[87] in a fluid medium containing 40 per cent chicken serum for the first fifteen days and 40 per cent horse serum thereafter. ×45.

B. Third passage chick embryo cells cultivated for eight days in a medium containing chicken serum, then for twenty-one days in a medium containing horse serum. ×45.

A grade of cellophane that is commonly used is Dupont's PT (plain, transparent) 62, thickness about 40 microns. It is usually supplied in rolls 3½ ins. wide, and may be perforated* in a variety of different patterns. One type of perforation that has been found satisfactory provides a regular staggered pattern of 324 holes per sq. in., each hole 0.023 in. in diameter, giving an open area of 13½ per cent. The cellophane is cut into squares to fit the thimble of a Soxhlet extraction apparatus and extracted for one hour with acetone to remove surface impurities. The squares are then rinsed and stored in glass distilled water.

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* Beckley Perforating Co., Garwood, New Jersey.

Eventually the wet cellophane is cut to fit the culture flasks. Discs to fit the small D-3.5 Carrel flasks may be cut quickly and easily with a 35-mm. cork borer; a large rubber stopper serves as the cutting surface. Flasks containing cellophane are sterilized by autoclaving.

The tissue fragments are placed in the flasks, under the cellophane, a few minutes after the medium has been added. The fragments may be introduced with a pipette or a platinum spatula, or, they may be inserted with a pipette and oriented with a spatula. With D-3.5 flasks, 1.0 ml. of medium may be used; and, if it is to consist of natural ingredients, two parts horse serum, two parts Tyrode's solution and one part chick embryo extract (1:20) may be used for the tissues of most warm-blooded vertebrates, including the human.

The majority of the cells usually adhere to the under surface of the cellophane, rather than to the glass, and become anchored to the edge of the perforations. When the cellophane is unperforated, or when the tissues are cultivated directly on glass, stresses and strains eventually develop in the growing sheets of tissue, which often break loose, degenerate and wash away. As shown by Evans and Earle,[87] better growth is obtained when the tissue fragments are placed under the perforated cellophane, rather than over it. These authors suggest that the cells may thrive best under the cellophane because of more favorable local conditions or because they are less likely to be washed away. Very often, the cells grow in several overlapping layers, and, when the medium is adequate, they continue to multiply until the whole surface of the cellophane has been covered. There is no limit to the size of the flask that may be used, and its shape is purely a matter of individual preference.

Flask Techniques for Continuous Circulation of Fluid Media

The first attempt to furnish tissue cultures with a continuous supply of fresh nutrient medium was made by Burrows,[29] in 1912. By means of a cotton wick, the fluid medium was carried from a reservoir at one end of the system, through a culture chamber, and then discharged into a receiving reservoir placed at a lower level. The tissues to be cultivated were fixed with plasma to an open network of cotton fibers prepared by teasing apart a portion of the wick. Since this early work of Burrows many attempts have been made to increase the volume of medium in relation to the mass of tissue without preventing cell respiration. The first practical step in this direction was taken by Carrel,[35] in 1923, when he introduced the flask techniques. Eventually, some of the flasks were constructed with lateral, wing-like extensions[40] for the accommodation of still larger amounts of culture fluid. When these flasks were rocked on platforms kept in constant motion with a windshield wiper (operated by suction), the fluid was caused to flow back and forth over the coagulum containing the tissues.

For certain types of experiments it seemed desirable to increase still further the relative volume of the culture medium. But techniques had to be devised if this was to be accomplished without impairing cell respiration. More particularly, a means had to be found to cause a large volume of fluid to flow evenly over the culture and at the same time to maintain a relatively constant *p*H and provide an adequate supply of oxygen.

In 1928 de Haan[120] described an apparatus for the circulation of a fluid medium through a culture cell that could be placed under the microscope. But the apparatus was rather complicated and consisted of many separate parts, some of which were connected with rubber tubing. A few years later Carrel[40] designed for the purpose a pump flask consisting of a single glass unit that was easy to clean and sterilize. But because the circulation of the fluid in the pump flask was actuated by two glass check valves that often stuck, the device was not entirely satisfactory. In 1939, however, Lindbergh[170] devised a flask for the cultivation of a large quantity of tissue in a thin layer of well-oxygenated medium that circulated continuously. Unfortunately, however, neither the Carrel pump flask nor the Lindbergh flask provide for microscopic examination of tissues during cultivation. But for certain types of experiments, such as the study of cell secretions, the behavior of tissues in synthetic media, and the propagation of viruses, the Lindbergh flask can be used to definite advantage. As much as 1 gm. of tissue can be cultivated in 30 to 80 ml. of medium, and samples of both the tissue and the medium can be removed very easily during the course of an experiment.

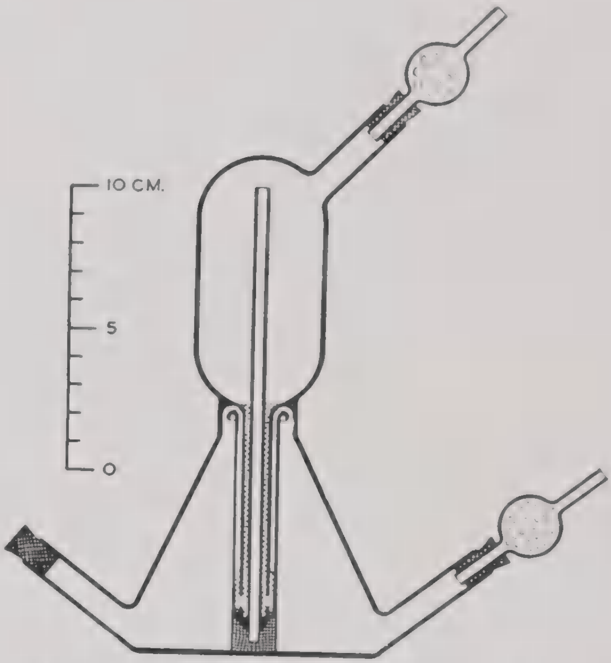

FIG. 54. Lindbergh culture flask for the continuous circulation of a large quantity of fluid medium. For description, see text. (C. A. Lindbergh.[170])

The Lindbergh flask (Fig. 54) is made of a single piece of Pyrex glass and consists of two chambers, one above the other. The main bulk of the culture medium is contained in the upper chamber (reservoir chamber), whereas the lower chamber (culture chamber) is for accommodation of the tissue. Circulation and oxygenation of the culture medium is actuated by a very slow flow of control gas through the cotton-filter bulb leading to the culture chamber. This gas passes through an inner glass tube leading from the bottom of the culture chamber to the top of the reservoir chamber and out through the cotton-filter bulb which protects the opening of the reservoir chamber.

This flow of control gas is unobstructed as long as the fluid level in the culture chamber is below the opening of the inner glass tube (about 2.5 mm. from the bottom of the culture chamber). With the flow of control gas unobstructed, the fluid medium from the reservoir chamber passes slowly down through fine silica sand in the middle glass tube, out through holes at the bottom of the tube, up through the outer glass tube to the lip at the top and down the inner wall of the culture chamber, until it reaches the fluid layer at the bottom of the culture chamber. When the fluid level in the culture chamber rises sufficiently to close the opening at the bottom of the inner glass tube the control gas entering the culture chamber slowly raises the pressure in the chamber. This pressure forces the fluid medium up in the inner glass tube and down in the outer glass tube. The fluid level continues to drop in the outer glass tube until it reaches the sand at the bottom of the tube. The fluid level continues to rise in the inner glass tube until it reaches the top of the tube and overflows into the reservoir chamber. After the fluid level in the culture chamber touches the bottom of the inner glass tube, the fluid maintains contact with this tube until the fluid level in the culture chamber has fallen appreciably below the bottom of the tube. Consequently, the depth of fluid covering the tissue fragments is constantly changing during the cycle of operation of the flask. Minimum and maximum depths of fluid are dependent on the distance of the bottom of the inner glass tube from the floor of the culture chamber. A cylindrical platinum screen extends from the floor of the culture chamber to the bottom of the outer glass tube. The bottom of this screen, which prevents fragments of tissue from passing into the inner glass tube, is sealed into the glass floor of the culture chamber.

When the fluid level in the culture chamber drops far enough to break contact with the bottom of the inner glass tube, the pressure of control gas in the chamber quickly lifts the column of fluid left in the inner glass tube and carries it into the reservoir chamber. As soon as the inner glass tube is open again, and the culture chamber is back to atmospheric pressure, the fluid in the reservoir chamber begins to flow through the silica sand in the middle glass tube, up over the lip of the outer glass tube and down the inner wall of the culture chamber, thereby completing the cycle of circulation.

To operate the flask, about 2 ml. of control gas is required per minute. The cylinder containing the desired mixture of gases (e.g., 8% CO_2, 21% O_2 and 71% N_2) is placed in a convenient location near the flasks. A pressure gauge and a needle valve are attached to the cylinder outlet. A rubber tube connects the needle valve with a saturation bottle partly filled with water. The outlet of the saturation bottle is connected to a glass T-tube. One arm of the T-tube leads to two bottles that are connected in series and partly filled with water to serve as a pressure-regulating valve for the control gas. The remaining arm of the T-tube leads to a condensation bottle and, beyond that, to a series of glass T-tubes (one for each flask) connected one with the other and with the condensation bottle by rubber tubing. Each T-tube is attached to a capillary glass (thermometer) tube of very fine bore, approximately 20 cm. in length. These capillary tubes are connected with the culture flasks by rubber tubing attached to the cotton-filter bulbs leading to the flasks.

The saturation bottle should be high enough to permit the control gas to be thoroughly saturated with water vapor as it bubbles through. The two bottles that serve as a control gas pressure-regulating valve should be high enough to cause the control gas to bubble through a total of approximately 25 cm. of water. The condensation bottle is simply an empty bottle placed between the saturation bottle and the capillary glass tubes to prevent the possibility of water getting into the latter. The apparatus will operate properly as long as there is any bubbling through the pressure-regulating bottles, regardless of how slow this is.

The medium should be placed in the flask (about 10 ml. in the lower chamber, the balance in the upper chamber) and the flask attached to a gas cylinder for adjustment of the *p*H before the tissue is added. For certain types of experiments a layer of perforated cellophane may be placed in the bottom of the flash. Moist cellophane can be inserted through one of the side-arms and flattened with a platinum spatula before the flask is sterilized (by autoclaving).

CHAPTER XII

ROLLER-TUBE TECHNIQUES

IN 1913 Carrel[32] cultivated tissues in tubes lined with a thin coagulum of plasma and embryo tissue extract. As a direct development of these early tube techniques, Carrel* later (1929) devised cylindrical culture chambers to fit a motor-driven rack. When the rack containing the culture chambers was revolved in a constant-temperature water-bath, the fluid phase of the culture medium flowed at a uniform rate over the coagulum containing the tissue.

The first practical roller-tube system of cultivation was devised by Gey[115, 116] and is now employed by many investigators for the long-continued propagation of normal and malignant tissue strains. According to the technique most commonly used, the cultures are carried in Pyrex test-tubes that are rotated continuously in a nearly horizontal rack accommodated in an incubator. The rotation provides a slow washing action that promotes an adequate exchange of food materials and waste products between the fluid and the cells. The rack is driven by a motor to give from six to fifteen revolutions per hour, and is so tilted that the fluid bathing the cultures does not come in contact with the stoppered ends of the tubes. The tissues may be cultivated directly on the walls of the tubes in a fluid medium (Fig. 57), or they may be planted in a thin coagulum of plasma (Fig. 55) or fibrinogen. The two methods and the various types of equipment that are used will now be described in some detail.

SPECIAL ROTATING DEVICES

According to the original technique of Gey, thin-walled Pyrex test-tubes (without rim, 150 mm. long, 15 mm. outside diameter) are fitted with Standard No. 0 rubber stoppers. The rotating tube carrier, capable of carrying ninety or more tubes, consists of three hexagonal plates fixed 2 ins. apart to a central rod, which projects through the back of the incubator. Two of the plates are drilled with suitable holes for the tubes. One inch from the back plate there is a copper screen to protect the ends of the tubes thrust against it; the tubes project out about an inch in front of the rack. The central rod is turned by a motor with reduction gears to give six revolutions per hour.

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* Carrel, A. Unpublished experiments.

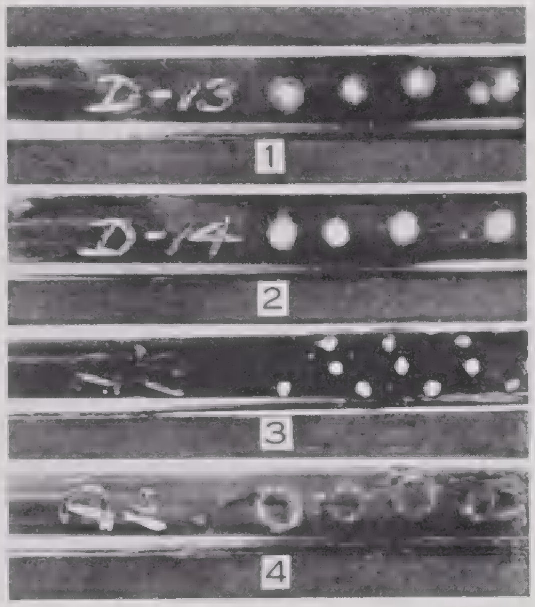

FIG. 55. Four-year-old strain of human sarcoma cells cultivated in roller tubes for twenty-nine days (1 and 2); for eleven days (3); and, for eleven days after the central portion of the cultures had been transplanted to another tube (4). (G. O. Gey and M. K. Gey.[116])

Some years later, Coman and Stabler[60] devised a very inexpensive type of apparatus which consisted of a motor and tube carrier mounted to form one complete unit. The motor, obtained from a General Electric clock having an automatic starting mechanism, was detached from the clock and removed from its housing. The exposed gears were removed until there remained only the gear of the main drive-shaft and two gears connected with it. One of these was found to turn at a speed of nine revolutions per hour. A piece of sheet brass, cut so as to leave this gear exposed, was fastened to the face-posts of the clock, and a small hole drilled through this sheet supported the end of the shaft of the intermediate gear. The sheet of metal also served to fasten the motor to its mount, which supported one end of the tube carrier. The tube carrier, constructed from plywood and drilled with eighty-four holes, was mounted on the frame of a bicycle pedal. The unit was silent in operation, produced no vibration and was easily transported.

Shaw, Kingsland and Brues[238] devised a roller-bottle system of cultivation in which a hole 33 mm. in diameter was drilled in the wall of an ordinary commercial, flat-walled, 2-ounce bottle. A coverglass was cemented over the opening with a plastic cement that was nontoxic and insoluble in water after prolonged dry heat sterilization (overnight at 160° C.). One cement* that was used was soluble in alcohol; another† was soluble in acetone. The cultures were prepared on the surface of the coverslip, which was treated with plasma, and fluids were added as soon as the clot had hardened. The cultures were incubated without motion, or were rotated from six to fifteen revolutions per hour. A rotating device accommodating twenty-four bottles was driven by a small synchronous-type electric motor (Telechron C-2 or C-4). A motor with a shaft speed of 1 r.p.m. required a 4:1 reduction gear.

Ott, Tennant and Liebow[203] devised a unit for rotating either a simple test-tube holder or a Novy jar. By means of the latter, a controlled atmosphere could be provided for the cultures.

Porter, Claude and Fullam[217] devised a special culture flask with a long, straight neck to fit a roller-tube rack. This flask, with two parallel flat sides 35 mm. in diameter, is blown from a regular Pyrex tube measuring 15 × 160 mm.

In the author's laboratory, two roller-tube carriers are in use. A large one, designed to hold 352 tubes, is accommodated in a general constant-temperature (37° C.) room, and a smaller one that will hold 144 tubes is accommodated in its own special incubator (Fig. 56). Each is driven by a ¼ h.p. motor with a

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* Gelva, supplied by Shawinigan Resins Corporation.

† Glyptal Number 1202, supplied by General Electric Company.

900:1 reduction gear to give twelve revolutions per hour. Because the motor for the larger carrier is also accommodated in the constant-temperature room, the thermostat controlling the heating units for the room has to be altered slightly on occasions when the unit is not in use, because of the heat generated by the motor. The motor for the small, self-contained unit is placed outside the incubator.

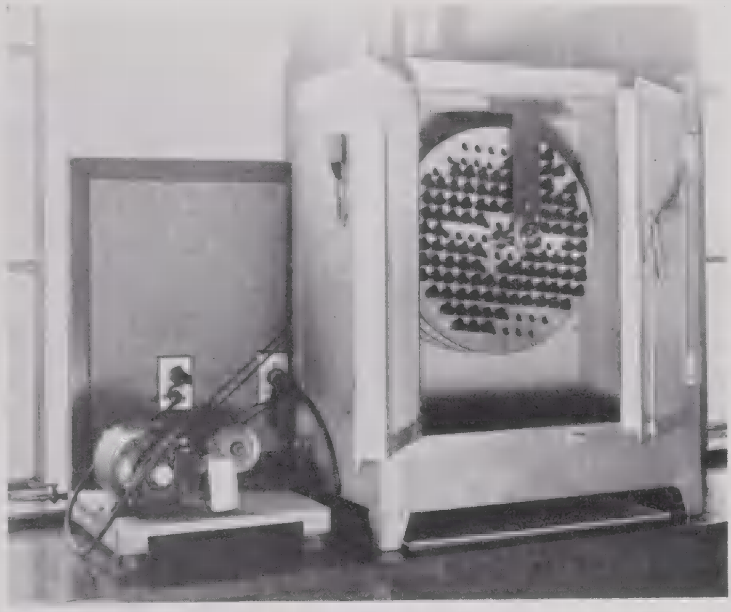

Fig. 56. A small roller-tube incubator with rotator and motor assembly.

The small tube carrier is 19 ins. in diameter, the large one 29 ins. in diameter. Each is constructed from three plywood plates, only the first two of which are drilled with holes for the tubes, which project out about 1¼ ins. in front of the rack. In each instance, the shaft is ball-mounted and is rotated by a tight-fitting v-belt connected with the motor assembly. Also, by releasing a small spring device placed on the shaft, one may spin the carrier at will in order to arrange and select the tubes. The test-tubes that are used are 18 mm. in outside diameter and 150 mm. in length, and they are fitted with No. 1 rubber stoppers. While these tubes have a greater diameter than those ordinarily used, the curvature of the wall is less and it is easier to photograph the cultures (Fig. 57).

ROLLER-TUBE CULTURES WITHOUT PLASMA

While it is true that a plasma (or fibrinogen) coagulum provides the most adequate anchorage for cells, there are many undertakings in which a plasma coagulum cannot be used. Thus, for example, in investigations having to do with the development of a synthetic medium it is not possible to employ a protein supporting structure of any sort (see Chap. VIII). For this purpose, the only alternative to the use of plasma, at present, is to use plain glass surfaces, or glass surfaces covered with perforated cellophane (see p. 188), bathed with fluid. Almost all types of cells will grow on glass, though some grow more

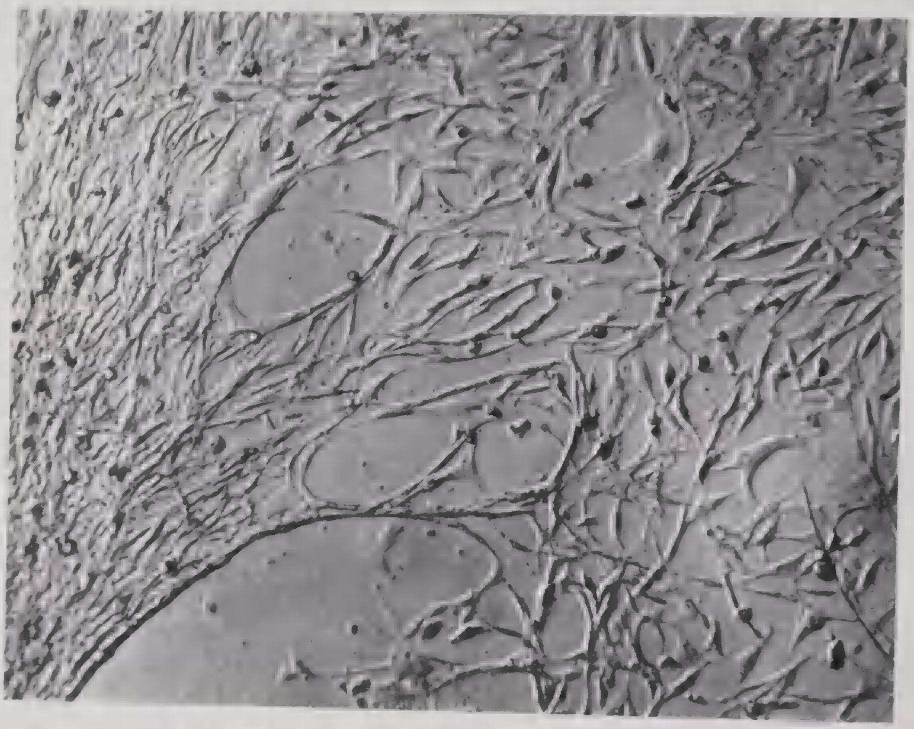

FIG. 57. Cells from an eleven-day-old chick embryo cultivated for sixty-nine days in a roller tube directly on the glass with a fluid medium consisting of 40 per cent horse serum, 40 per cent Earle's salt mixture and 20 per cent chick embryo tissue extract. ×75.

abundantly than others. But few long-continued test strains that are carried in serial passage grow as vigorously under these conditions as embryo tissues freshly isolated from the organism.

It is extremely easy to initiate a luxuriant growth of tissue cells in roller tubes without a coagulum. Thus the cells from one or two small fragments of chick embryo skeletal muscle will continue to multiply in a fluid medium until all of the tube that is bathed with the medium becomes lined with a dense growth. If the medium consists of two parts horse serum, two parts Tyrode's solution or its equivalent, one part embryo tissue extract (1:20) and 0.005 per cent, or less, of phenol red to serve as an indicator of *p*H, the cultures can be kept healthy and active for several months. From time to time, as the density of the growth increases, local areas of stress develop and whole sheets of cells become detached from the glass. Eventually, these detached cells degenerate and are washed away, and new cells grow into the vacated areas. Other cell masses, that degenerate while still attached to the glass, may be scraped off with a spatula.

In the preparation of roller-tube cultures without plasma, the medium is first placed in the bottom of the tube. The tubes are held in an inclined rack, such as a wooden block drilled with holes at a 45° angle and holding about twelve tubes (Fig. 17). The tissue fragments are then introduced, along one side of the tube, by means of a long platinum spatula (Fig. 25) or a pipette with a tip that is bent at a slight angle (Fig. 22). If a pipette is used, care must be taken to remove the excess fluid carried over with the tissues; or, a spatula may be used to move the tissues to a dry area once they have been deposited on the glass. If the tissue fragments are left to dry, very slightly, before they are flooded with the nutrient fluid, they will remain adherent to the glass, that is, if their first contact with the fluid is brought about with the utmost caution. But the fragments should be flooded with fluid before the tubes are placed in the incubator. Usually, a ten- or fifteen-minute drying period will suffice. During the drying period, it will be possible to adjust the *p*H of the medium, if it seems too alkaline. The adjustment may be accomplished by gassing each tube with a mixture of 8 per cent carbon dioxide, 21 per cent oxygen and 71 per cent nitrogen while it lies, almost horizontal, in a small metal frame. During this operation, the tube is so oriented that the tissue fragments are not in contact with the medium.

The fluids may be renewed two or three times a week, depending upon the activity of the cultures. The old fluid, or a measured portion of it, is withdrawn with an extra-long hand-drawn pipette fitted with a rubber nipple. When it is desired to prepare transplants for fresh tubes, they may be cut from tissue scraped from the walls of the tube with a platinum spatula.

Roller-Tube Cultures with Plasma

The preparation of roller-tube cultures imbedded in a plasma coagulum may be carried out by following essentially the same techniques that have already been described for flask cultures (see Chap. X). Four or five drops (about 0.3 ml.) of pure plasma are introduced and spread with the end portion of a curved-tip pipette (Fig. 22) to cover the lower two-thirds of the tube, which is kept horizontal. The tissue fragments, of which there may be twenty or more, are then selected with a curved-tip pipette, concentrated at the extreme tip of the pipette to free them of excess fluid, and deposited in the upper, plasma-free portion of the tube. After the excess fluid has been removed with a capillary pipette, the fragments are oriented in the plasma layer and the excess plasma is allowed to drain to the bottom of the tube. At this point about six or seven drops (about 0.4 ml.) of diluting fluid are introduced and mixed with the plasma that has collected in the bottom of the tube. This diluting fluid may consist of four parts of placental cord serum or horse serum, four parts of balanced salt solution (Tyrode's solution or its equivalent) and two parts of embryo extract. Or any combination of these or similar materials may be used, depending upon the nature of the tissue and the purpose of the experiment. Once the diluting fluid has been mixed with the plasma in the bottom of the tube, the new combination is distributed over and between the fragments that have been placed in the thin plasma layer on the walls of the tube, and the fragments requiring it are quickly reoriented. If all this is done with the utmost despatch, a very thin coagulum will be obtained. But the diluting mixture, which is prepared beforehand, must be mixed with the excess plasma at the bottom of the tube and spread over the walls of the tube a few seconds after the tissue fragments are first oriented in the undiluted plasma. If a thick coagulum is required, the amounts of the various materials to be used should be worked out in preliminary tests and the tubes should be rotated until the materials have begun to coagulate. The amounts will depend on the size of the tubes that are used.

The tubes are fitted with rubber stoppers and left lying on the work table until it is quite certain that the coagulum is firm enough to withstand a supernatant fluid, which may consist of 2 ml. of the same materials that were used to dilute the plasma. Once the supernatant fluid has been added, it is spread over the coagulum by a gentle rolling and tipping motion and the tubes are placed in the rotator.

Some cultures may digest the coagulum fairly rapidly and require frequent patching. If the digestion is not too extensive fresh plasma may be introduced and spread over the coagulum after the old fluid has been withdrawn; and fresh fluid may be introduced after the new plasma layer has coagulated. Otherwise a fresh diluting mixture may be introduced, mixed in the bottom of the tube with fresh plasma, and the combined mixture spread over the old coagulum by rotating the tube until clotting occurs. If the tube is rotated beyond a certain point, however, the new coagulum will separate from the old and the whole procedure will have to be repeated until the new coagulum remains attached.

At 37° C., the supernatant fluid should be changed two or three times a week. But if the cultures are incubated at a lower temperature (32° C., for example), cell growth will be greatly diminished and the fluid may be changed less often, perhaps as infrequently as twice a month.

CHAPTER XIII

FLASK TECHNIQUES FOR TISSUE FRAGMENTS IN SUSPENSION

WHEN it is desired to keep tissues alive for limited periods, such as four or five days, in a state of functional survival, with little or no proliferation of their constituent elements, they are chopped to extreme fineness and placed in 125-ml. Erlenmeyer flasks in 3 ml. of fluid medium, the depth of which is just sufficient to cover the tissues. This system of cultivation provides an ideal method for the simultaneous study of the effect of the medium on the cells and the effect of the cells on the medium. Thus the entire medium may be changed without removing the suspended fragments, or any part of it may be withdrawn at any time to test it for the presence of particular substances elaborated by the tissues. Some or all of the suspended fragments may be fixed, sectioned and stained for morphological study.

The first tissues to be cultivated in suspension in a fluid medium were used for the propagation of viruses. In 1928 Maitland and Maitland[182] employed a suspension of minced kidney tissue in serum and Tyrode's solution for the cultivation of vaccinia virus. Since then many viruses have been propagated in tissue suspensions cultivated in a wide variety of media. In the early experiments little attention was given to the degree of survival of the tissue elements. It is certain, however, that some of the cells must have remained alive long enough to promote virus multiplication. The virus yield was often high, and as yet no instance is known of the propagation of virus particles at the expense of dead cells.

CULTIVATION OF TISSUES IN SUSPENSION FOR PHYSIOLOGICAL STUDIES

In 1936 the author[210] undertook a study of antibody formation in which an attempt was made to discover the conditions favoring the survival of adult tissues in suspension, for limited periods. Each of the cultures contained fifty or more fragments of adult rabbit spleen that were cultivated in autologous and homologous sera under a variety of special conditions. The tissues were cut into relatively large fragments of such a size that it required about seventy-five to weigh 100 mg., and the cutting was done with cataract knives on a glass plate. The medium consisted of three parts rabbit serum, one part isotonic sodium bicarbonate (1.4 per cent), two parts Tyrode's solution containing four times the usual amount of glucose, and 0.005 per cent phenol red to serve as an indicator of *p*H. Because demonstrable antibodies were produced by the tissues during the period of cultivation, and because it was possible to fix,

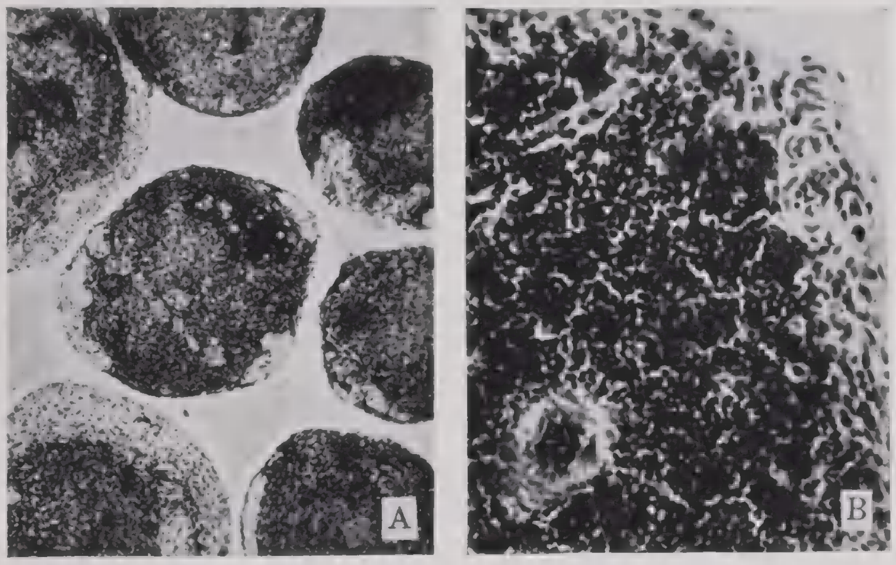

FIG. 58. *A* and *B*. Stained sections of fragments of adult rabbit spleen cultivated for four days in a fluid medium containing 50 per cent rabbit serum. In each instance, approximately 100 mg. of tissue were suspended in 2 ml. of medium. *A*, ×42; *B*, ×230.

section and stain the tissue fragments from each culture at the termination of an experiment, it was easily possible to correlate the degree of survival of the tissues with at least one measurable, functional activity. And while it was found that spleen fragments from different animals might remain in an equally good state of preservation when cultivated in a given serum, their antibody production might vary tremendously. But a high antibody production was never demonstrated in cultures that showed widespread necrosis of the tissue elements, particularly of the lymphocytes. Certain sera were decidedly toxic, whereas others permitted the tissue to remain in a state of preservation that was well-nigh perfect (Fig. 58). Oddly enough, however, instances were encountered in which a serum from another animal provided a better culture medium than autologous

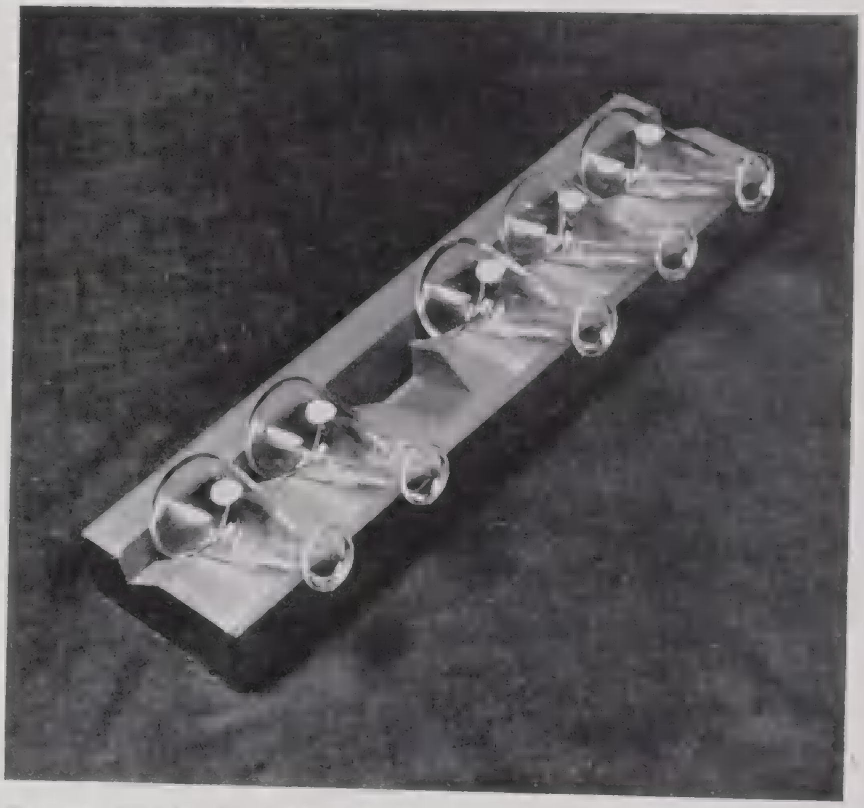

FIG. 59. Wooden block for the accommodation of Erlenmeyer flasks being filled with sterile media.

serum from the animal providing the tissues. Whenever possible, therefore, it is wise to use a variety of sera in all preliminary experiments.

Until a synthetic medium has been elaborated, blood serum or its equivalent must be present if the tissues are to be kept in good condition even for limited periods. The serum should comprise not less than one-third and not more than one-half of the medium. Excellent results have been obtained* by cultivating mouse endometrium (Fig. 61*C* and *D*) in 125-ml. Erlenmeyer flasks in 3 ml. of medium consisting of one-third heated (56° C. for one hour) rabbit serum and two-thirds Simms' salt mixture. It is probable, however, that equally satisfactory results might have been obtained by using horse serum or human placental cord serum, instead of rabbit, and by substituting one of the other salt mixtures now in common use. Human tissues may be cultivated either in human serum or in horse serum. Horse serum, in fact, provides an excellent

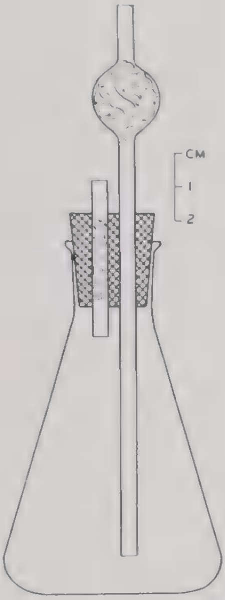

FIG. 60. 125-ml. Erlenmeyer flask arranged for preliminary or continuous gassing of culture in which tissue fragments are suspended in thin layer of fluid medium. For description, see text. (R. C. Parker and A. J. Hollender.[211])

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* Parker, R. C. Unpublished experiments.

nutrient for tissues from many sources, even including the chick.

The medium is placed in the flasks and is gassed to an appropriate *p*H (see p. 153) before the tissues are added. While the flasks are open they are placed on their sides in a wooden rack (Fig. 59) to minimize the danger of bacterial contamination from falling dust particles. It is now common practice to prepare the tissues by mincing them on a glass plate; and it is almost impossible to have them too finely divided. The mincing is done with cataract knives, with curved scissors, or with the halves of double-edged razor blades held in tightly-fitting hemostats or surgical needle holders (Fig. 21). A coarse-tipped pipette is used to transfer the tissue from the cutting-plate to the flask.

When Erlenmeyer flask cultures are to be gassed continuously, the openings are fitted with two-holed rubber stoppers accommodating two glass tubes (Fig. 60). One of these tubes extends almost to the bottom of the flask and is surmounted by a cotton filter bulb attached directly to the gas line. The other tube is for escaping air; it is short and plugged with cotton. When the cultures are not to be gassed continuously, the special gassing device is used for adjusting the initial *p*H of the medium and is then replaced by a solid rubber stopper. Certain tissues with high oxygen requirements may require continuous gassing. In this event, the gas mixture is led into the flasks through the same sort of pressure-regulating system described on page 194.

As already indicated, the tissue fragments may eventually be fixed, sectioned and stained for histological study. Just prior to fixation, the fragments from each culture flask are collected in a compact mass arranged on the surface of a solid plasma or fibrinogen clot prepared on a glass slide. The mass of fragments is then covered with a protective layer of fresh plasma and extract (or thrombin), and as soon as this layer has coagulated the whole mass is immersed in fixing solution. The material may be left on the glass slide until it is ready for embedding (see p. 250).

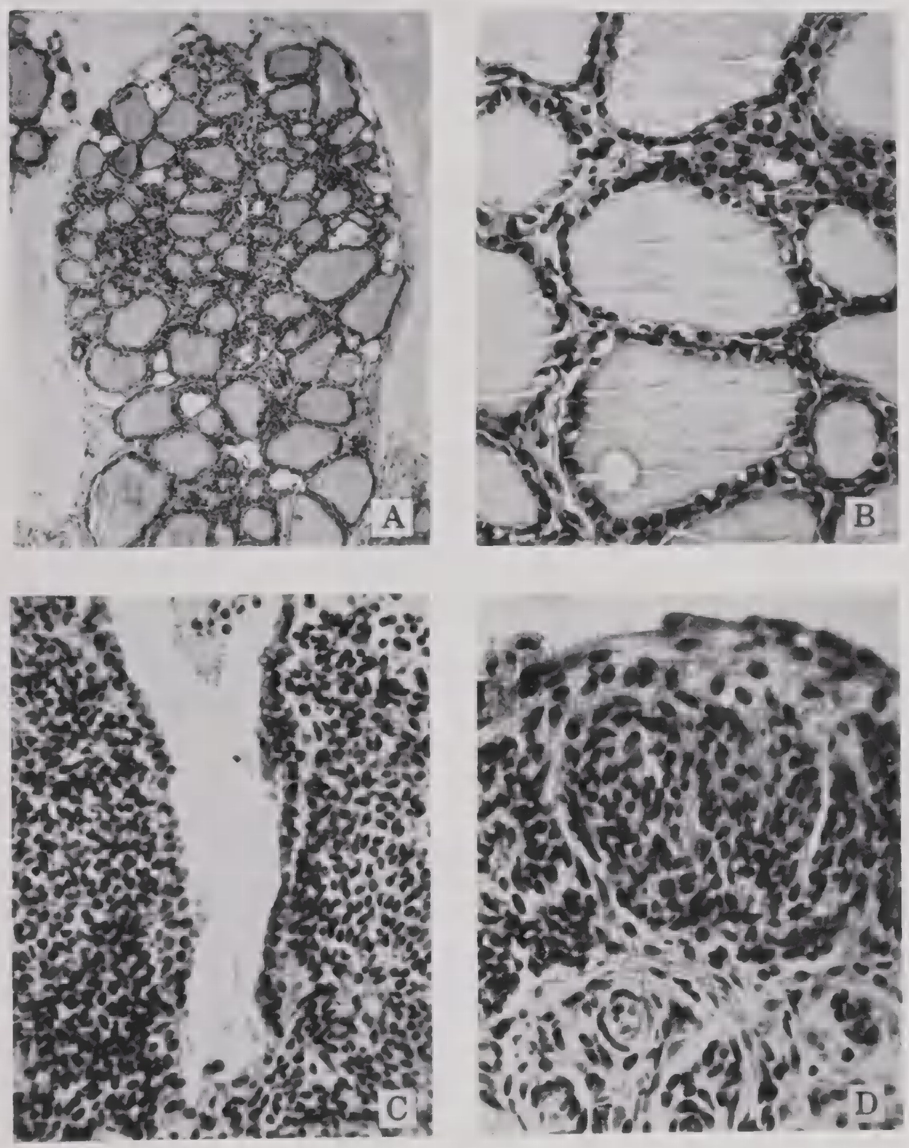

Fig. 61. *A* and *B*. Stained sections of fragment of adult rabbit thyroid cultivated for five days in a fluid medium containing 50 per cent rabbit serum. Approximately 100 mg. of tissue were suspended in 2 ml. of medium. *A*, ×62; *B*, ×230.

C and *D*. Stained sections of fragments of uterus from an ovariectomized mouse cultivated for three days in 125-ml. Erlenmeyer flasks in 3 ml. of fluid medium, approximately one-third of which was rabbit serum. *C*, ×270; *D*, ×340.

For many types of experiments having to do with the functional abilities of cells and tissues, apart from their ability to multiply, it would seem that this is the ideal method of cultivation. Most of the more specialized cell functions (for instance, hormone secretion) are detectable only when the cells are in a state of comparative rest, that is, when they are not undergoing active proliferation. And because the method just described is an exceedingly simple one that requires little or no special equipment, it could also be used to advantage by investigators who might hesitate to use more complex procedures. Thus, by studying stained sections of tissue fragments after twenty-four or forty-eight hours' cultivation, one would be able to determine, very simply, the direct effects of toxic substances on a wide variety of cell types.

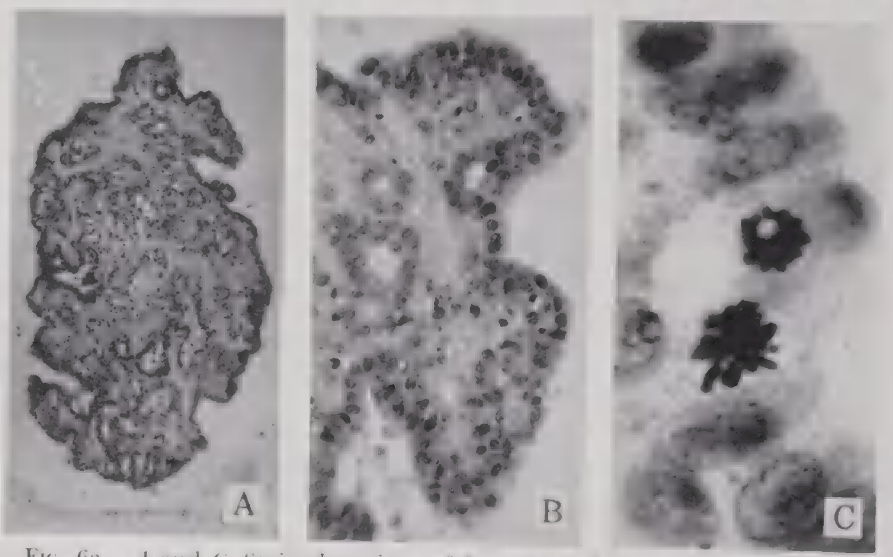

FIG. 62. *A* and *C*. Stained sections of fragment of human breast tumor after four days' cultivation in a 125-ml. Erlenmeyer flask in 3 ml. of fluid medium, one-third of which was human serum. The flasks were gassed continuously with a mixture of 21 per cent oxygen, 8 per cent carbon dioxide and 71 per cent nitrogen. *A*, ×80; *B*, ×245; *C*, ×900.

CULTIVATION OF TISSUES IN SUSPENSION FOR VIRUS PROPAGATION

Some years ago an attempt was made by the author and his associates[211, 212] to determine the optimal conditions for the

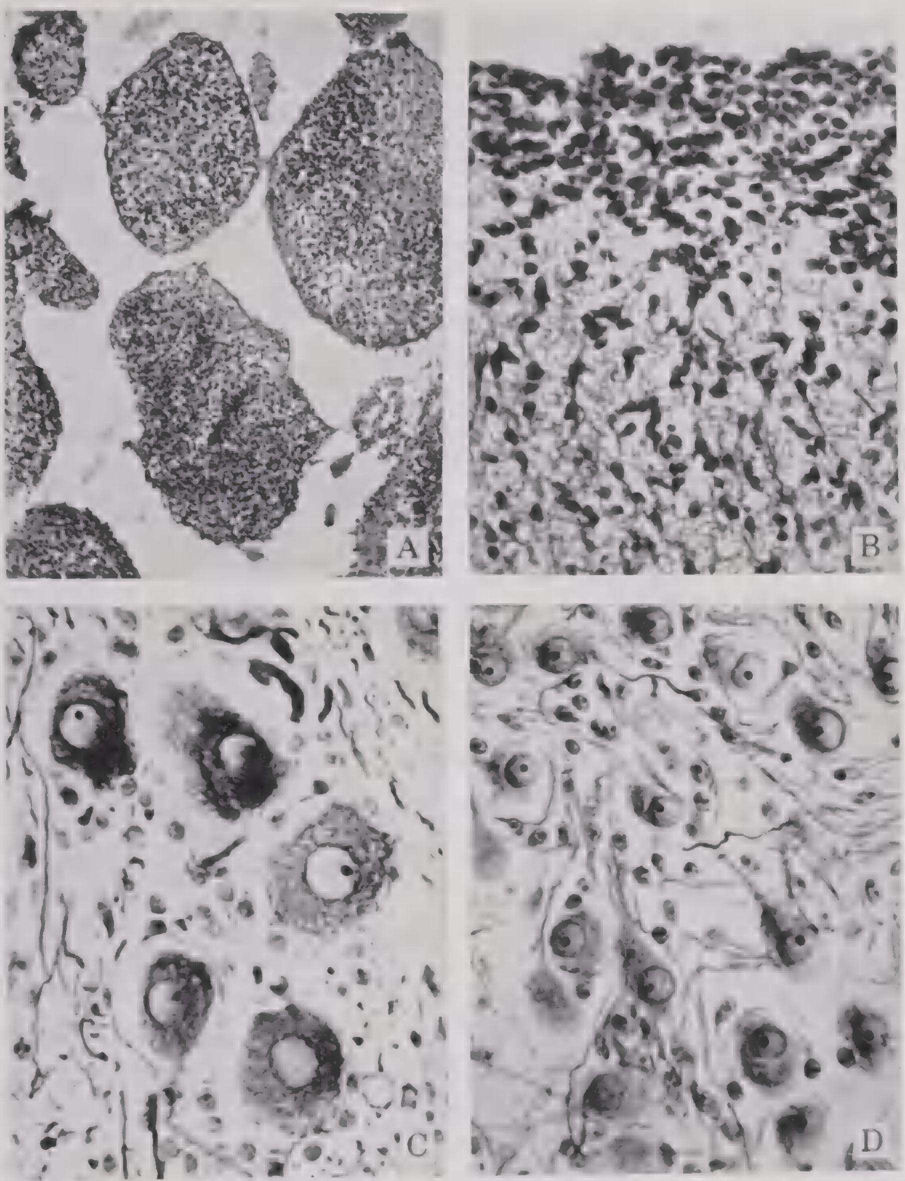

FIG. 63. *A*. Stained section of fragments of embryo mouse brain after three days' cultivation in a 125-ml. Erlenmeyer flask in 3 ml. of fluid medium consisting of ox serum ultrafiltrate (⅓) and Simms' solution (⅔). Bodian's silver stain. ×77.

B. Fragment from a similar culture after two days' cultivation. ×340.

C. Stained section of fragment from a mixed culture of monkey nerve tissue (olfactory bulbs, sympathetic ganglia and Gasserian ganglion) after three days' cultivation in a Lindbergh flask (Fig. 54) in a medium containing 25 per cent monkey serum. Gassed continuously with 40 per cent oxygen, 8 per cent carbon dioxide and 52 per cent nitrogen. ×412.

D. Stained section of a fragment from another culture similar to that described in *C*. ×412.

propagation of Theiler's GD-VII virus of mouse encephalomyelitis and the rabies virus, although the latter had been cultivated previously by several investigators.* Because these viruses were cultivated in brain tissues, which are among the most difficult to keep alive, under these conditions, the techniques that were used and the results obtained will be described in some detail in the hope that they may serve as a guide to the cultivation of other viruses with less exacting requirements.†

For both viruses (rabies and Theiler's GD-VII), the tissue component of the medium consisted of finely chopped embryo mouse brain, one large brain or its equivalent being used for each culture (Fig. 63*A* and *B*). The medium consisted of rabbit serum (1/3) or ox serum ultrafiltrate[240] (1/3) diluted with Simms' solution (2/3). Before the tissue was added, the medium was adjusted to *p*H 7.1 or 7.2 by gassing for one or two minutes with a mixture of 8 per cent carbon dioxide, 21 per cent oxygen and 71 per cent nitrogen. For this purpose the flasks were closed with the special gassing device shown in figure 60. If the cultures were not to be gassed continuously, the gassing device was replaced by a solid rubber stopper after the tissue and the virus inoculum were added. As an indicator of *p*H, all cultures contained 0.001 per cent phenol red.

When the original virus inoculum was prepared from brain material that had been preserved in 50 per cent glycerine in saline, the glycerinated tissue was cut into coarse fragments and washed three times in great excess of glucosol. (Virus material stored in a frozen state was weighed and ground without washing.) It was then weighed, ground in a mortar with coarse sand (60 to 80 mesh) and made up to 1:10 with distilled water. This dilution was allowed to stand for one hour in an ice bath before higher dilutions were prepared in beef broth containing 10 per cent ox serum ultrafiltrate. The 1:100 dilution was ordinarily used for the initial inoculation of the culture

* Kanazawa, K. (1936); Webster, L. T., and Clow, A. D. (1936); Kligler, I. J., and Bernkopf, H. (1938); and Plotz, H., and Reagan, R. (1942).

† The reader is also directed to an interesting report by J. P. Fox (*Am. J. Hyg.*, 1947, *46*, 1) on the factors influencing the multiplication of yellow fever virus (17D) in tissue culture.

series; this and higher dilutions were used for mouse (Swiss strain) titrations to determine the potency of the original virus. For each culture the inoculum was 0.3 ml.; for each mouse the intracerebral injection was 0.03 ml. Three mice were injected at each of several dilutions. After three or four days' incubation, the culture virus was transferred to fresh brain cultures, and mouse titrations were made to test its killing power.

For each virus (rabies and Theiler's GD-VII), comparative experiments were made to test its relative behavior under the following conditions: when cultivated in rabbit serum and in ox serum ultrafiltrate, when incubated at 35° C. and at 37.5° C., when the cultures were incubated with and without continuous gassing, when the tissue material used for subculturing and for animal testing was ground and resuspended in the medium, or when the cell-free medium was inoculated without grinding the tissue. The results of these and other tests will now be given.

Theiler's GD-VII virus was propagated for 20 three or four-day passages (transfers) in cultures of chopped mouse embryo brain suspended in ox serum ultrafiltrate (1/3) and Simms' solution (2/3). After an initial series of culture passages, the cell-free culture fluid killed mice in dilutions as high as 1:1,000,000 when 0.03 ml. was injected intracerebrally. Eventually, the same results were achieved when the ox serum ultrafiltrate was replaced by heated (one hour at 56° C.) rabbit serum. The virus multiplied more abundantly at 35° C. than at 37.5° C.; and, the yield was higher in 125-ml. Erlenmeyer flasks containing 3 ml. of medium than in 50-ml. flasks containing 4 ml. of medium. No increase in virus output was ever obtained by inoculating subcultures with ground material; nor did grinding the culture tissues improve the mouse titrations. Also, no real advantage was gained by subjecting the cultures to continuous gassing with mixtures of oxygen, carbon dioxide and nitrogen. The concentration of virus in the culture medium was higher after two days' incubation than after one day. After two days the yield diminished. Culture virus from twentieth-passage embryo mouse brain cultures that had been frozen at −60° C. for forty-nine days was propagated successively, though at reduced titer, in cultures of brain tissue from one-day-old mice (five passages), three-day-old mice (three passages), five-day-old mice (four passages) and fourteen-day-old mice (one passage).

When the rabies virus was propagated for fifty-seven passages (transfers) in cultures of chopped mouse embryo brain suspended in rabbit serum (1/3) and buffered salt solutions (2/3), under conditions developed in shorter experiments, the virus multiplied to the extent that mice were killed regularly by the culture material in dilutions as high as 1:1,000,000, and it was possible to produce typical rabies in mice with 1/33,000,000 ml. of the culture virus. When ox serum ultrafiltrate was substituted for rabbit serum, the virus titer was somewhat less. Comparative experiments also showed the importance of grinding the culture material for mouse titrations. Tissues to be ground were separated from the medium and reduced to a fine paste with TenBroeck grinders that were kept chilled in an ice-bath during the grinding. After low-speed centrifugation, the ground material was resuspended in the cell-free supernatant. But no significant increase in virus output was ever obtained by subjecting the cultures to continuous gassing with mixtures of oxygen, carbon dioxide and nitrogen. A duplicate series of cultures incubated for 9 passages at 35° C. and at 37.5° C., respectively, showed no consistent difference in the amount of virus produced. Attempts to cultivate the rabies virus in chick embryo brain cultures were unsuccessful even when the virus had been adapted to chick tissue by serial intracerebral passage in embryonated eggs. In contrast, however, Plotz and Reagan[214] have reported the propagation of the street rabies virus in cultures of whole chick embryo tissue in a medium consisting of Tyrode's solution (3 ml.) and monkey serum (0.5 ml.) to which three drops of chicken plasma were added to form a floating clot.

CHAPTER XIV

WATCH-GLASS CULTURES FOR EMBRYOLOGICAL STUDIES

TISSUES cultivated *in vitro* show two types of growth, unorganized and organized. Unorganized growth resembles wound healing. When the medium is rich in growth-stimulating substances, cells from the explant migrate out into the medium with great rapidity and multiply to form a broad halo of undifferentiated cells. Organized growth, on the other hand, corresponds more nearly to normal growth in the body. When it occurs, the explant enlarges as a whole, the outward migration of cells from the explant is reduced to a minimum, the normal histological structure is preserved, and, if the explant is in an early stage of development, it usually continues to differentiate histologically, and sometimes anatomically as well (Fell[88]). Furthermore, if the conditions are appropriate, organized growth may occur in the interior of the explant while unorganized growth is taking place at the margin.

The watch-glass method of cultivation was originally devised by Strangeways as a means of studying organized growth. Since then the method has been perfected by Fell[88, 89] and her associates, and has been used to excellent advantage by them as a means of supplementing the standard morphological methods of studying the developmental potencies of undifferentiated embryonic regions. Briefly, the method consists in placing the explant on the surface of a plasma coagulum prepared in a 1½-in. watch glass which, in turn, is placed in a 3-in. Petri dish containing a circular mat of absorbent cotton that is moistened with sterile water to prevent evaporation (Fig 64). The medium usually consists of equal parts of plasma and embryo extract. At intervals of 48 hours, the entire explant is removed from the clot, to which it adheres by the outgrowth of superficial cells, and is washed and transferred to a freshly prepared watch glass. In this way the further development of the explant may be observed for a period of several weeks (Fig. 8).

The watch-glass method has the great advantage that the explant is not embedded in the medium, but can be freely manipulated on the surface of the clot. And because of the size of the container, relatively large explants can be grown. As Fell[88] points out, however, the maximum size to allow further growth and normal development *in vitro* depends largely upon the nature of the tissue. Thus, it is possible to cultivate a fragment of cartilage 3 to 4 mm. in diameter, provided it is not too thick, whereas an epithelial explant more than 2 mm. in diameter soon becomes necrotic in the interior. Fell has also shown that when a relatively large rudiment is to be cultivated, the limiting factor of size can often be overcome by using embryos of a smaller species.

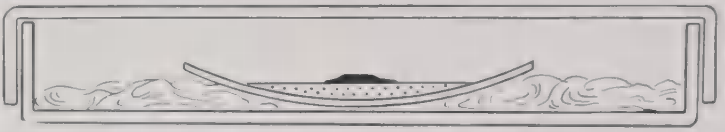

FIG. 64. Cross-sectional diagram of a watch-glass culture. For description, see text. (H. B. Fell.[88])

The dissections are made with a fine cataract knife and an ordinary fine sewing needle broken off short and mounted in a thin glass rod. The knife as it comes from the makers is useless for most experiments, and the point has to be specially ground to extreme sharpness and flexibility by the worker himself. The needle is also sharpened before use. When the instruments have been properly prepared very fine dissections of early rudiments can be made and explants not more than 200 microns in diameter can be excised from certain regions with almost perfect precision. The dissections are made in a drop of saline in a depression slide under an ordinary wide-field stereoscopic microscope. A magnification of 15 diameters for large structures and 24 diameters for smaller ones is usually employed.

Embryological experiments which do not depend upon a large series of individual cultures made simultaneously, or upon the long-continued cultivation of special strains, can be performed in any clean, draught-free room without all the architectural precautions usually prescribed for tissue culture. It is only necessary to attach a small glass canopy to the microscope stage to prevent contamination from falling dust particles. In work of this nature, one good experiment is an achievement that usually justifies the effort of sterilizing the glassware and other materials that are used. Yet one should not feel too badly about an occasional contamination. In most tissue culture laboratories, contaminations happen with appalling frequency despite the most elaborate precautions.

CHAPTER XV

GROWTH MEASUREMENTS AND THEIR INTERPRETATION

THE growth of cell colonies cultivated *in vitro* has been measured in several ways: (1) by making periodic measurements of the surface area of the cultures and constructing diagrams in which the increase in area is expressed as a function of time; (2) by estimating, for a particular period of time, the ratio of resting cells to dividing cells in a given area; (3) by weighing the cultures; and (4) by measuring certain chemical changes that take place in the culture system. These various methods will now be discussed in some detail.

MEASUREMENT OF SURFACE AREAS

The method commonly used in measuring surface areas was devised by Ebeling[83] in 1921. According to this procedure, an image of the culture, magnified from 10 to 15 times its actual size, is projected on paper by means of a projectoscope. The general contour of the periphery is traced in pencil. As often as necessary, the culture is again projected on the same sheet of paper and at the same magnification as before. If the culture has increased in size, a new outline is made showing where and to what extent the colony has become larger. The surface area enclosed by each successive outline is measured with the planimeter and appropriate curves or diagrams are constructed.

If a projectoscope is not available one may resort to a simpler procedure that was in general use in Carrel's laboratory in the early days of tissue culture. At that time[81] it was the practice to estimate the approximate area of a culture by measuring various axes with the ocular micrometer of a microscope at low magnification, and then compute the area according to the formula, πr^2. Swift, Moen and Vaubel[246] made a comparative study of the two methods and found that ocular micrometer estimates agree fairly closely with those derived by measuring projectoscopic drawings with a planimeter. The main disadvantage in computing areas from ocular micrometer measurements is the amount of time often consumed in making unnecessary estimates. Significant differences can always be detected by inspection without any measurements whatsoever. And since many experiments, particularly those of a preliminary nature, are never reported in the literature, considerable time may often be saved by making projectoscopic drawings as a matter of routine without measuring the actual areas until it is necessary to record them for publication. If a planimeter is not available, the drawings may be measured with a hand-rule, to establish an approximate radius for estimating the area.

It requires very little imagination to realize that this simple means of determining the activity of cell colonies must have certain limitations. Surface measurements make no distinction between increases in area resulting from cell multiplication and increases due to cell migration, though both phenomena usually contribute to the ultimate size of the cell colony.[267] Nor do surface measurements take into account variations in the density of the cell population, in the general thickness of the colony, or in cell size. Still, the estimation of surface area has given significant data in thousands upon thousands of experiments. But like every other measuring device, it must be used with discretion and with a complete understanding of the possible sources of error. The method should *not* be used under any of the following conditions:

1. *when the cultures to be compared are grown in hanging drops.* Such cultures cannot be kept in good condition for much longer than forty-eight hours without being transferred to fresh media; for so short a period and under conditions so difficult to standardize, quantitative measurements of the rate of increase in surface area are quite meaningless.

2. *when the cultures to be compared are grown in flasks of*

unequal size or in flasks containing unequal quantities of medium.

3. *when the cultures to be compared are grown directly on glass in a fluid medium.* For uniform growth and migration, the supporting structure of a plasma or fibrinogen coagulum is necessary.

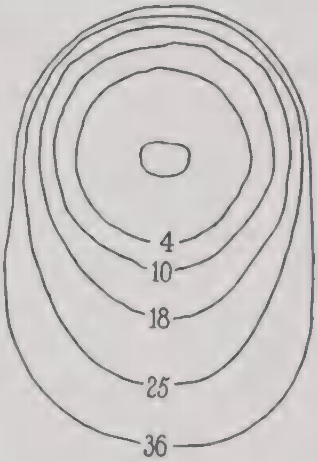

FIG. 65. Diagram showing the increase in surface area of a colony of fibroblasts belonging to a strain that had been isolated from the skeletal muscle of a fourteen-day chick embryo and cultivated in plasma and Tyrode's solution for thirty-six days. Note the increasing irregularity in the contour of the culture as a result of localized cellular activity. (R. C. Parker.[206])

4. *when the peripheral areas of the cultures vary greatly in the density of the cell population.*

5. *when the central areas of the cultures vary greatly in thickness.*

6. *when the cultures digest the fibrin of the plasma coagulum too quickly for compensation by regular "patching" with superimposed clots.*

7. *when the cultures consist of tissue fragments explanted directly from the organism.* The cell population of each fragment is peculiar to the particular region from which it has been taken; no two regions in the body are identical.

8. *when the cultures are comprised of cells heterogeneous in type and appearance.* Each type of cell has its own specific food requirements and expresses its functional capacities in

different ways; and the various cell types may not be present in the same relative proportions in the cultures to be compared. An exception to this rule is made in the case of leucocytes; adjacent fragments cut from a single layer of leucocytes obtained from the buffy coat of whole blood can usually be assumed to contain the same cell types in the same relative proportions.

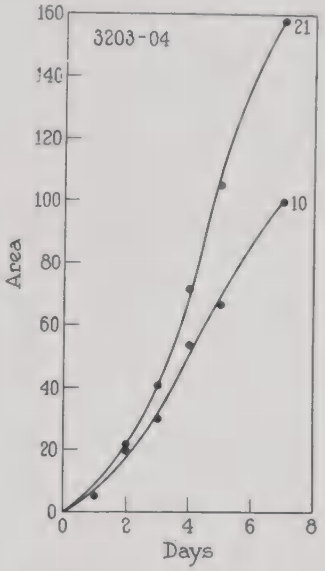

FIG. 66. Curves showing the increase in surface area of colonies of fibroblasts representing two strains isolated simultaneously from the *M. tibialis anterior* of a ten and a twenty-one-day-old chick embryo, and cultivated for fifty-two days (nine passages) in chicken plasma and chick embryo tissue extract. (R. C. Parker.[207])

9. *when the cells are of such a type as to produce an uneven, irregular growth.* Thus, for example, cultures of epithelium may grow either in membranes comprised of a single layer of cells or in dense pavements of many layers; again, certain groups of cells from these cultures may grow in membrane formation, whereas others may form cords and tubules.

10. *when the cultures to be compared are cultivated in a medium that tends to reduce the rate of cell migration to a minimum.* As a result of local conditions in the central regions of the tissue mass or at certain points on the periphery (Fig. 65), the configuration of such cultures often becomes very irregular.

It follows, therefore, that interpretations based upon surface measurements must be made with extreme caution. In reporting a series of such measurements, it is essential to show that each of the foregoing conditions has been carefully avoided. In addition, end differences of less than 10 per cent of the surface area of the colonies should never be used to substantiate positive results. Even under the best conditions, there may be an experimental error as great as 20 per cent incident to the material itself. At the termination of an experiment it should be possible to detect significant variations with the unaided eye. Besides, it must be remembered that the facility with which the cells wander out into the medium is quite independent of actual growth, though it often happens that actively growing tissues also show active cell migration.[267] In short,

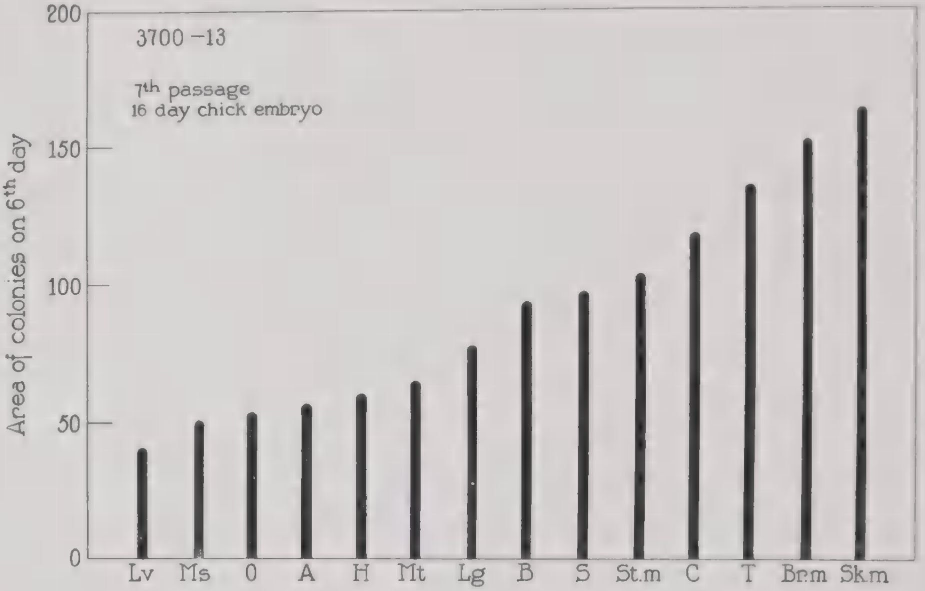

FIG. 67. Diagram showing the increase in surface area of colonies of fibroblasts representing fourteen strains isolated from the liver (*Lv*), mesonephros (*Ms*), ovary (*O*), aorta (*A*), heart (*H*), metanephros (*Mt*), lung (*Lg*), bone (*B*), spleen (*S*), stomach musculature (*St.m*), cartilage (*C*), thyroid (*T*), breast musculature (*Br.m*), and skeletal musculature (*Sk.m*), of a sixteen-day chick embryo, and cultivated in a mixture of plasma and embryo tissue extract; from planimetric measurements of the areas attained by the colonies on the sixth day of the seventh passage. (R. C. Parker.[208])

surface measurements are not growth measurements. Growth has to do only with the production of new protoplasm.

It is unnecessary to keep the cultures at incubator temperature during the actual process of making surface measurements. If all the cultures of a given series are withdrawn from the incubator for the same length of time, they will show the same relative activity over the period of interrupted incubation. Cultures will not die if they are left at room temperature for hours at a time, or even for days.

There are various ways of presenting surface measurements. Occasionally it may seem desirable to reproduce the original tracings (Fig. 65). Where detailed measurements of a large number of cultures are to be reported, they may be listed in the form of a table. Whenever possible, a few typical experiments should be selected for graphic presentation, either in the form of curves (Fig. 66) or of simple diagrams (Fig. 67). Curves are seldom more informative than line diagrams and require much more space, particularly when comparisons can be made on the basis of end values alone.

Estimation of Mitotic Coefficients

Another means of measuring the rate of cellular activity is to estimate, for a particular moment of time, the ratio of dividing cells to resting cells and to express this ratio in terms of "mitotic coefficients" (Champy).[54] If, for example, M represents the number of dividing cells counted and C the number of resting cells found in the same area, then $M/C \cdot 1000$ is considered as the *mitotic coefficient.*

This method has certain obvious advantages and certain disadvantages. It does serve to distinguish between cell migration and cell multiplication (mitosis) in the particular area or areas studied, and for a given cell type under a given set of environmental conditions, the ratio of dividing cells to migrating cells in the peripheral areas is probably surprisingly uniform from culture to culture. It also serves to detect differences due solely to variations in the density and thickness of the colonies. But unless the architecture of the colonies is completely uniform from culture to culture, and unless it is realized that mitotic counts taken in any particular area at any particular moment do not in any way express the activity of the culture as a whole,[110, 268] the counts can be very misleading. It is known, for example, that mitotic activity is greatest at the periphery of the culture, where the cell population is of optimum density.[268] If mitotic counts are made on fixed and stained material, a large number of cultures must be prepared for each variable under comparison in order that representative cultures from each group may be removed from time to time for fixation.

Willmer[268] and Willmer and Jacoby[271] have described an elaboration of this technique that has yielded much new information. In principle, the procedure consists in photographing directly, on 3-inch cine-bromide paper, a sample of cells (100 to 400) from the peripheral part of a colony of fibroblasts or macrophages. The photographs are taken automatically at six-minute intervals for varying lengths of time up to five days[142] and estimates are later made of the percentage of dividing cells, per unit of time, according to the formula

$$\tfrac{1}{2}\left(\frac{x}{a+(b-x)}\right)\times\frac{100}{t}$$

when a is the number of cells present at the commencement, b those present at the end, x those that divide during the period, and t the time in hours. The value obtained is expressed as the *mitotic index*.

MEASUREMENT OF DRY WEIGHTS

For a long time it was believed impossible to weigh cultures because of the difficulty in separating the cells from the plasma coagulum. Eventually, however, Meier[190] succeeded in determining the dry weight of fibroblast cultures used in a study of lactic acid production. The fluid phase of the culture medium was removed and replaced with enough 98 per cent alcohol to make the total concentration 10 per cent of the entire medium. The culture was then incubated at 37° C. for one or two days, or until the plasma coagulum was completely dissolved. As a result, the intact tissue colony remained either floating in the fluid or else adhering to the bottom of the flask. The contents were then shaken out into a glass dish, the tissue was transferred with a platinum spatula to a small fragment of weighed glass coverslip, and the preparation was dried at 100° C. until repeated weighings on the microbalance were constant.

In order to determine the weight of transplants at the beginning of the experiment, Meier prepared control flasks from halves of each of the cultures placed for incubation. As soon as the clots containing these halves were firm, they were treated with alcohol and eventually removed for weighing in the manner already described. By weighing, in a single flask, four halves of the same size and origin, he was able to approximate very closely the weight of the individual transplants in each experiment. Thus, the dry weight of four such halves was estimated to be 0.015 mg. After six days' incubation, the same number of sister fragments weighed from 0.11 to 0.16 mg. according to the nature of the medium.

A similar technique was later devised by Laser,[156] who digested the plasma coagulum with pepsin. When the clot was covered with concentrated pepsin (Grübler), the complete culture could be removed almost instantly in its entirety. The time required to free the culture was found to vary with the nature of the plasma. Consequently, the action of the pepsin had to be watched very closely. When it seemed desirable to slow down the process of digestion, the pepsin was diluted.

Lipmann[172] found that cultures could be freed from plasma by adding a weak solution of sodium hydroxide until the final concentration in the medium reached 0.25 N. The plasma clot dissolved within one or two hours at room temperature, and the tissue could then be removed, washed with distilled water, and weighed on the microbalance.

Using the dry weight techniques, Meier[190] and Laser[158] have compared the increase in the weight of cultures with the increase in surface area. They were able to prove conclusively a long-suspected fact, namely, that the increase in surface area is due largely to cell migration, not to cell growth and multiplication. Laser was further able to show that the true growth of cultures as determined by an increase in mass continues even when the increase in surface areas is negligible; and further, that the increase in weight does not really become appreciable until cell migration comes to a standstill. Laser also found that fibroblasts grown in serum may show a greater increase in weight, and hence, more growth, than sister cultures grown in embryo extract. On the fourth day five cultures placed in tissue extract had an average surface area of 85.3 sq. cm., those in serum, an average area of only 18.7 sq. cm. But the average weight of the serum cultures was 0.15 mg., as compared with 0.12 mg. for those grown in tissue extract. The expression "slow growth" must, therefore, be used with extreme caution. Although tissues grown in serum may remain small and inconspicuous, they keep fair pace in actual mass increase with those grown in substances producing more active migration.

Measurement of Total Metabolism by Manometric Methods

For many years the early tissue culture workers searched for some practical physiological means of evaluating the activities of cells *in vitro*. Eventually, the cell metabolism investigations of Warburg and his associates offered a promising approach to the problem and the first studies of this nature were conducted in Warburg's own laboratory. Thus, in 1927 Warburg and Kubowitz[261] investigated the respiratory metabolism of excised heart fibroblast cultures suspended in chicken serum. They compared freshly isolated fibroblasts with fibroblasts of Carrel's old strain, which had then been transplanted three thousand times, and found that the metabolism was highest in the cells from freshly isolated cultures. It decreased progressively during three to four culture passages and thereafter remained constant. Wind,[274] another member of Warburg's laboratory, carried out a series of metabolic investigations on cultures of Rous chicken sarcoma, heart fibroblasts, iris epithelium, and spleen tissue. His investigations included the influence of bicarbonate and sugar concentrations on glycolysis, the determination of lactic acid and glucose, and the nature of growth under anaerobic conditions.

Since it had long been known that surface measurements fail to distinguish between cell multiplication and cell migration, a series of attempts were then made to measure growth by determining the increase in metabolism. The first experiments in this direction were made by Meier,[189] who used Warburg manometers to study the oxygen consumption of fibroblast cultures. Lipmann[171, 172] and Laser[156, 157] both carried forward this work; each of them made his own special adaptations of the original Warburg procedures and succeeded in measuring simultaneously, and without interrupting the experiment, the oxygen consumption, and the aerobic and anaerobic glycolysis.

By making manometric determinations of the rate of anaerobic glycolysis of chick heart fibroblasts at the beginning and at the end of a period of nine days (four passages), Lipmann and Fischer[173] were able to show that the original quantity of tissue had increased twelvefold. In a subsequent investigation Lipmann[172] found that fibroblasts from bone showed much greater aerobic glycolysis than those from the heart. Furthermore, the energy derived from glycolysis represented the minimum necessary to support growth, for under anaerobic conditions, the growth of heart fibroblasts practically ceased.

On the basis of total metabolism experiments with fibroblasts Laser[156] decided that it was impossible to draw any conclusions regarding the actual amount of increase in the mass of the cultures. He found, for example, that oxygen consumption per unit of weight continued to increase during the course of the experiment, whereas the anaerobic glycolysis decreased. In addition, aerobic glycolysis was not always constant. In other experiments[157] normal connective tissue cells survived for a considerable period (four to six days) anaerobically; when respiration was suppressed with nitrogen or with potassium cyanide, the increase in weight was the same as in oxygen. Laser concluded, therefore, that cells could grow and multiply anaerobically for a certain period at the expense of glycolysis.

More recently, Gemmill, Gey and Austrian[114] have studied the metabolism of a pure strain of malignant cells from Walker Rat Sarcoma 319. The tissues were cultivated in small cylinders fitted into roller tubes, with a medium consisting of plasma and serum. After four to ten days of growth, the cylinders containing the tissue were transferred to special metabolism chambers and were immersed in buffered Ringer's solution containing glucose. Measurements of oxygen consumption were then carried out over a twenty-four-hour period. The Qo_2 value (per mg. of dry weight per hour) of 4.5 was considerably lower than the figure of 10 to 22.9 reported by Warburg and Kubowitz[261] for fibroblasts, of 20 to 32 reported by Lipmann[172] for heart fibroblasts, and of 7.8 to 25.6 reported by Laser[156, 157] for osteoblasts. Victor and Lewis[253] also studied Walker Rat Sarcoma 319 and reported figures closely approximating those of Gemmill, Gey and Austrian. The low values obtained by these two groups of investigators were attributed to the use of phosphate Ringer's solution, rather than plasma or serum, while the metabolic measurements were being carried out.

Measurement of Glucose Utilization

Chemical changes occurring in the medium have been studied by many investigators in an effort to relate the metabolism of growing cultures to their increase in area and mass. In this connection, particular attention has been paid to the utilization of glucose. Willmer,[266] who studied the growth of embryo chick intestine in various saline mixtures, found that the total amount of cell migration was directly proportional to the glucose concentration up to 1000 mg. per cent. The tissues were more active, however, in concentrations of 200 mg. per cent as long as sufficient glucose was available. Willmer further observed that concentrations greater than 1000 mg. per cent appeared to be somewhat toxic. Increased growth with increasing glucose levels was also reported by Demuth[72] and by Ebeling.[85] The latter observed that the growth of fibroblast cultures increased as the glucose level was raised from 200 to 1150 mg. per cent. At higher concentrations growth was inhibited. Krontowski[149] found that tissues can use a total amount of glucose greater than their own dry weight in forty-eight hours, and Lebensohn[159] reported that the amount used decreased with the age of the animal supplying the tissue and, to a lesser degree, with the age of the animal whose plasma was used.

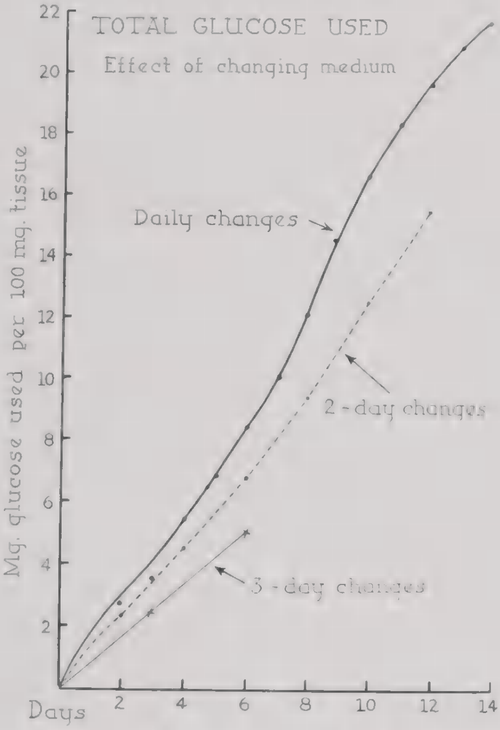

FIG. 68. Glucose utilization in roller-bottle cultures[238] containing weighed amounts of chick embryo tissue in plasma and Baker's solution,[8] without added serum. Cumulative curves showing total glucose used by one culture with daily renewals of fluid medium, and by two other cultures with the fluid renewed at longer intervals. (H. Wilson, E. B. Jackson and A. M. Brues.[273])

The whole problem of glucose metabolism in tissue cultures has been reinvestigated by Wilson, Jackson and Brues.[273] These authors cultivated chick embryo tissues in the semisynthetic feeding solution of Baker,[8] without added serum, and measured both the rate of glucose uptake from the medium and the total glucose utilized. They found that the initial rate of glucose utilization could be increased by increasing its concentration in the medium from 100 to 500 mg. per cent. Similarly, total utilization of glucose was at its highest level when the medium contained 300 to 400 mg. per cent. Glucose was found to be used in greatest amount when a medium containing 100 mg. per cent was replaced daily (Fig. 68). It was concluded that the amount of glucose used has no direct relation to growth. Cultures treated with colchicine in concentrations that blocked all mitoses continued to use glucose at the usual rate. Moreover, cultures grown in 500 mg. per cent glucose used more than those grown simultaneously in 100 mg. per cent, but examination of the fixed tissues at various periods showed about the same area of migration and similar mitotic counts in both groups. These findings were interpreted as an indication that the energy requirements for survival and growth are easily supplied, and that higher amounts of glucose do not necessarily increase the growth rate, which is probably limited by other factors such as permeability, rate of intracellular synthetic reactions, or availability of essential foodstuffs.

A somewhat different concept of the relation between glucose utilization and cell growth was developed by Pomerat and Willmer[216] in studies on chick periosteal fibroblasts. Respiratory poisons did not immediately stop mitotic activity, nor did conditions which interfered with the process of phosphorylation affect growth. From these observations, which were considered as indirect evidence, Pomerat and Willmer concluded that the essential process in carbohydrate breakdown was neither respiration nor glycolysis, but probably a direct glucose breakdown without preliminary phosphorylation. Earlier experiments by Lipmann,[171, 172] in which cultures were transferred to Warburg vessels and their glycolysis measured during growth, also appeared to show that glycolysis was not the characteristic source of energy for growth of tissues *in vitro*.

MEASUREMENT OF LACTIC ACID PRODUCTION

Aerobic glycolysis in tissue cultures was studied by Demuth and Meier,[73] who measured the lactic acid production. It was not possible, however, to correlate the amount of lactic acid formation with the increase in surface area. In a later investigation, Meier[190] determined the lactic acid production of four "slow-growing" cultures (in 2.5 per cent tissue extract) and four "fast-growing" cultures (in 15 per cent tissue extract), after he had computed their increase in surface area and measured their dry weights. The cultures grown in the smaller amount of tissue extract, and showing the least increase in surface area, gave the greatest lactic acid production per milligram of dry weight per day.

Krontowski[149] was able to account for about 60 per cent of the glucose used by his cultures as lactic acid, while Willmer[270] found no absolute correlation between high growth rate and high lactic acid production and noted that, in general, lactic acid formation followed the glucose consumption. Wilson, Jackson and Brues[273] further reported that lactic acid production was related in rate and amount to glucose utilization and observed that lactate was also produced during the period of regressive change after growth had apparently ceased. Under these conditions the amount of lactate evolved was less than in healthy cultures and tended to represent a greater proportion of the total metabolism. A small amount of lactate was formed in cultures not supplied with glucose; this was attributed to glucose formation through deamination of certain amino acids present in the medium. Some evidence was also obtained that tissues were able to utilize lactate and pyruvate after their glucose supply had become exhausted.

Measurement of Nitrogen Metabolism

The nitrogen metabolism of cultures growing in Baker's solution[8] has been studied by Wilson, Jackson and Brues.[273] The original amino nitrogen concentration of 14 mg. per cent in the medium increased by 24 to 31 mg. per cent after tissues had been cultivated in the solution for six days. This was considered to be evidence that fibroblasts hydrolyze Witte's peptone to smaller units by means of proteolytic enzymes as a preliminary step before these smaller units were utilized by the growing cells. Further experiments, in which an amino acid solution prepared by hydrolyzing Witte's peptone replaced the Witte's peptone in Baker's medium, showed that growing tissues could utilize simple amino acids. As evidence of this, the amino nitrogen of the solution was found to fall progressively during growth of the cultures.

MEASUREMENT OF NUCLEIC ACID PHOSPHORUS

A method of measuring the rate of cell multiplication in cultures by determining the amount of nucleoprotein phosphorus present in the tissues was introduced by Willmer,[270] who employed the chemical procedure of Berenblum, Chain and Heatley.[17] According to this procedure, measurements are made of the total phosphorus that is not extractable by the usual procedure of treating with hot alcohol and chloroform, followed by dilute acid. Willmer selected this means of estimating growth on the assumption that nucleoprotein is the most characteristic constituent of the nucleus, and that the greater part of the nucleoprotein is present in the nucleus. Later evidence[68] has shown that Willmer's assumption was incorrect, and it is now believed that there are two types of nucleic acid in each cell, the more abundant one usually being ribonucleic acid of the cytoplasm and nucleolus, the other, desoxyribonucleic acid, being confined, apparently, to the nucleus. Still, the method has come to be used quite extensively as a chemical means of determining growth in tissue cultures. Willmer[270] cultivated chick embryo heart tissues in plasma in roller tubes and found good agreement between nucleoprotein phosphorus and the dry weights of cultures as reported by Laser.[158] Also, Cunningham and Kirk[62] considered the nucleoprotein phosphorus method to be the best means that has yet been proposed for estimating growth.

Davidson and Waymouth[67] extended the work of Willmer by investigating the factors influencing the nucleoprotein content of fibroblast cultures. They were able to confirm Willmer's original observation that cultures maintained in Tyrode's solution alone decreased markedly in nucleoprotein phosphorus content over a twenty-four to forty-eight-hour period, and at the end of that time appeared to have reached a "steady state." The presence of embryo extract retarded, but did not entirely prevent, this loss of nucleoprotein phosphorus. The addition of embryo extract to cultures whose tissues were depleted of nucleoprotein by a maintenance period in Tyrode's solution alone resulted in a marked rise in nucleoprotein phosphorus. It was also observed that this rise occurred even after the extract had first been heated to 100° C. for five minutes. Later studies[70, 71] showed that chick embryo extract was more effective in increasing the nucleoprotein phosphorus content of the cultures than was sheep embryo extract with comparable nitrogen values, and that defatted plasma and extract gave essentially similar results.

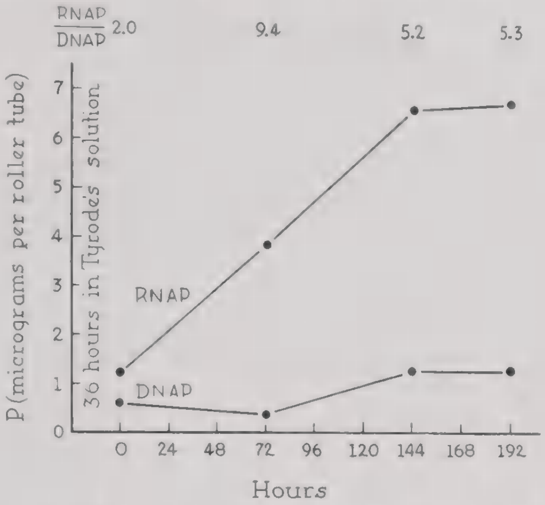

FIG. 69. Changes in ribonucleic acid phosphorus (RNAP) and desoxyribonucleic acid phosphorus (DNAP) in forty-eight small chick-heart explants growing in plasma clots in two roller tubes for 192 hours with daily renewal of medium containing chick embryo extract and fowl serum. (J. N. Davidson, I. Leslie and C. Waymouth.[66])

More recently Davidson, Leslie and Waymouth[66] employed the fractionation procedure of Schmidt and Thannhauser[236] to separate the nucleoprotein phosphorus into ribonucleic and desoxyribonucleic acids, and have followed changes in these two components in fibroblast cultures. When cultures were maintained in Tyrode's solution, the loss in nucleoprotein phosphorus was found to involve both the nucleic acids in the ratio of their occurrence in fresh tissue, and it was assumed that the decrease must arise from the breakdown and washing out of cells damaged in the process of preparing the explants. When the nucleoprotein content of the tissues was reduced to a minimum by maintaining the cultures in Tyrode's solution for twenty-four hours, after which they were supplied with daily changes of medium containing embryo extract and serum, the ribonucleic acid was found to rise sharply to about five times its initial value and to reach a steady level at about five days. During the same period the desoxyribonucleic acid remained unchanged for four days, then rose to about twice its initial value and showed no further increase (Fig. 69). It was observed that an increase in ribonucleic acid always preceded by relatively long intervals any increase in desoxyribonucleic acid, and this was interpreted[66] as evidence that ribonucleic acid is the precursor of desoxyribonucleic acid in growing fibroblasts. It was also observed that over a period of seven days the ratio of ribonucleic acid to desoxyribonucleic acid rose from an initial value of approximately 2.0 to a final value of about 5.2. This increase was attributed to the gradual predominance of fibroblasts over other types of cells originally present in the embryo heart explants. By adding colchicine to the cultures it was further established that the observed changes in the nucleic acid content of the cells were due to synthetic processes associated with the growth of new tissue. Colchicine, which has been shown[28] to arrest mitosis in tissue cultures, caused a fall in both ribonucleic acid and desoxyribonucleic acid even in the presence of embryo extract and serum.

It must be remembered that nucleic acid phosphorus determinations necessitate the destruction of the cultures; and in some instances it is necessary to pool several cultures in order to obtain sufficient material for analysis. The method does appear promising, however, as a means of establishing a chemical test for growth. Moreover, the refinement of the technique that renders it possible to estimate both ribo- and desoxyribonucleic acids has greatly extended its usefulness and has resulted in an accumulation of data that may be of value in establishing the role of these two nucleic acids in the process of cell division.[64, 65]

MEASUREMENT OF CHEMICAL CHANGES BY MEANS OF RADIOACTIVE ISOTOPES

Radioactive isotopes have also been employed in studies on the metabolism of tissue cultures. Cohn and Brues[58] cultivated tissues by their roller-bottle technique[238] and carried out continuous chemical measurements of the rate of exchange of isotopes between medium and cells. By this method they were able to study the permeability of tissue cells to potassium and phosphate ions, and to calculate the turnover time for these two elements.

Chapter XVI

HISTOLOGICAL PROCEDURES

THE preparation of cultures for histological study is relatively simple. According to the nature of the information desired, cells and tissues cultivated in flasks or tubes, or on coverslips, may be fixed and stained *in situ* or, when possible, embedded and sectioned. Because the fibrin of the coagulum has a marked tendency to overstain, most histological procedures have to be modified somewhat if cultures grown in plasma or fibrinogen clots are to be stained *in situ*. Even with a thin coagulum, it is usually difficult to obtain the necessary contrast between cells and medium. Sometimes the stain is almost completely removed from the cells during the process of dehydration, which must be greatly prolonged because of the density of the coagulum. When cultures are sectioned, these difficulties are eliminated. As already indicated on page 159, it is an easy matter to remove plasma cultures, intact, from the flasks prior to fixation. Or plasma cultures may be removed from the flasks during the early stages of fixation, before the clot has hardened (see p. 247). The latter procedure, which is recommended by Earle,[78] results in very little shrinkage.

Before proceeding, it should be pointed out that the present chapter is not intended as a general treatise on histological procedures, nor as a compendium of the innumerable staining procedures that have been applied to tissue cultures. Instead, an attempt will be made to set forth a few of the simpler staining techniques most satisfactory for general purposes. By following them, the beginner will not only acquire a certain degree of assurance in handling culture material but will also discover for himself the usual changes to be made in adapting any of

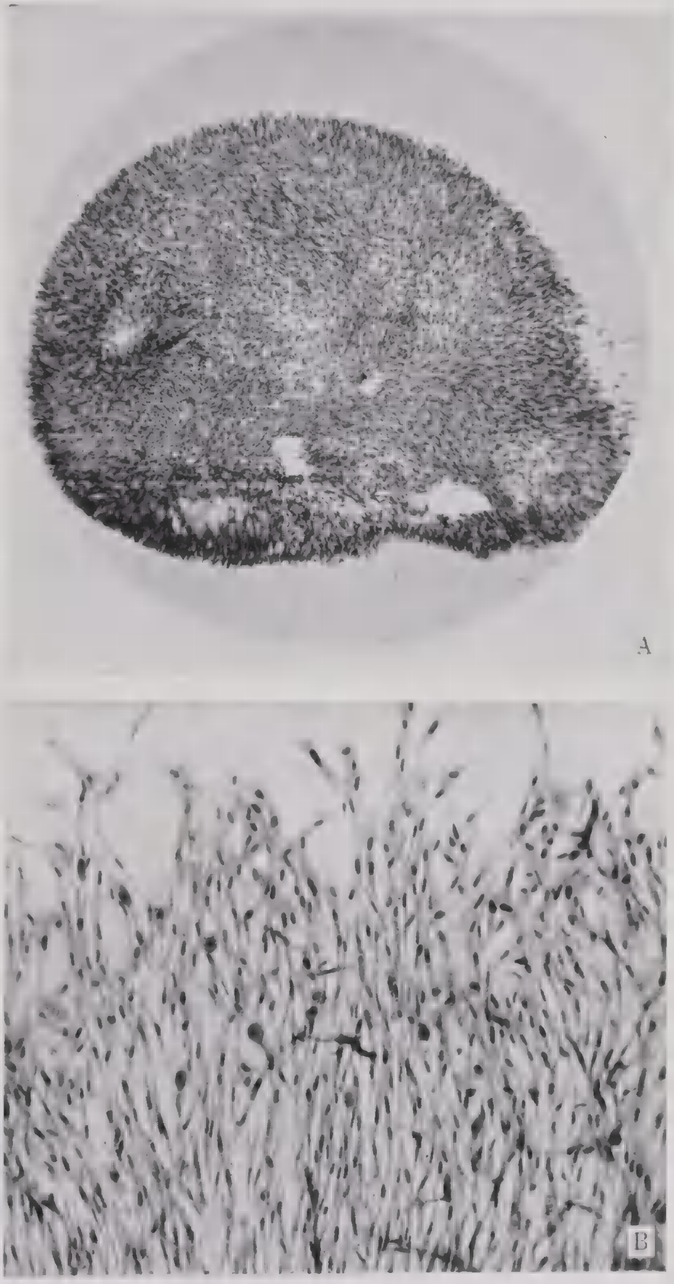

FIG. 70. *A*. Stained section of a hanging-drop culture of fibroblasts from bone. Hematoxylin-eosin after Zenker-formol. (A. Fischer and R. C. Parker.[110]) ×58.

B. Marginal area of a hanging-drop culture of fibroblasts from skeletal muscle; twelfth passage; hematoxylin after Ringer-formol. (R. C. Parker.[205]) ×175.

the procedures of histology to the special requirements of tissue culture.

In general either whole preparations or serial sections are made from flask, tube or coverslip cultures in which the living material is embedded in coagulated plasma or fibrinogen (Fig. 70). In addition, whole preparations are made from flask, tube or coverslip cultures in which the tissues are growing directly on the glass in a fluid medium (Fig. 71). And serial sections are made from flask cultures in which the living material consists of tissue fragments suspended in a fluid medium (Figs. 7 and 62).

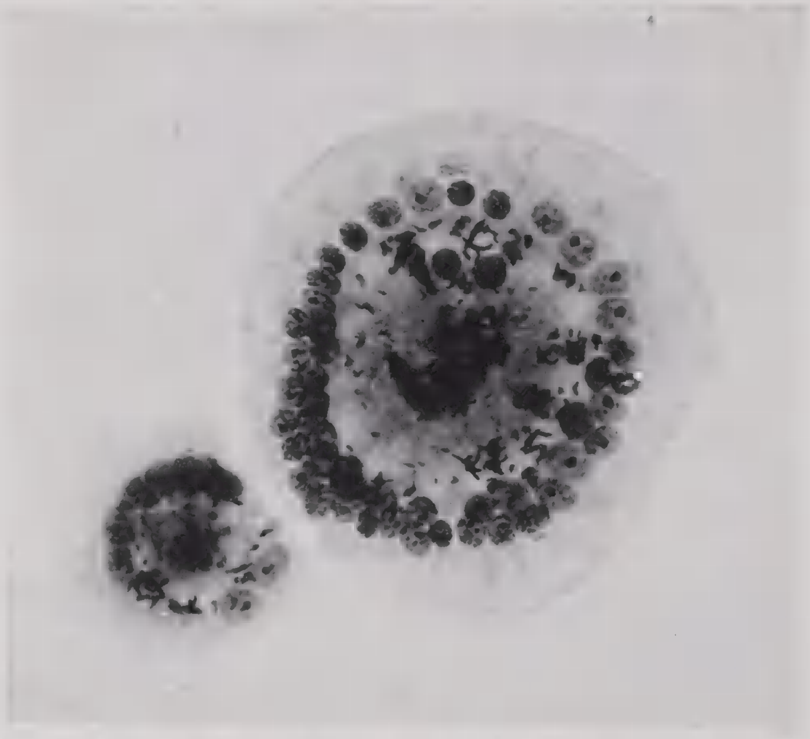

Fig. 71. Giant cell in a flask culture of chicken leucocytes infected with avian tubercle bacilli. Large aggregations of ingested bacteria are completely surrounded by nuclei. Harris's hematoxylin and Ziehl-Neelson's carbol-fuchsin after Ringer-formol. ×520.

In making whole mounts of coverslip preparations in which the living material is embedded in coagulated plasma, the culture is carried through the fixing and staining solutions on the coverslip and eventually mounted face down on a glass slide with neutral Canada balsam or clarite (see p. 248). When cultures are grown on mica coverslips instead of on glass, they are mounted somewhat differently, in order to reduce to a minimum the optical interference of the mica. After such cultures have been stained and cleared the mica sheets are split with a sharp knife or razor blade, in order to leave only a thin supporting membrane. The mica surrounding the culture is then trimmed away with scissors and the remaining square is mounted on a glass slide, culture side up, with a drop of Canada balsam between the slide and the mica. After a second drop of balsam has been placed on the culture itself, the whole preparation is covered with a thin glass coverslip (Fig. 72). Lead weights are applied during the final stages of drying.

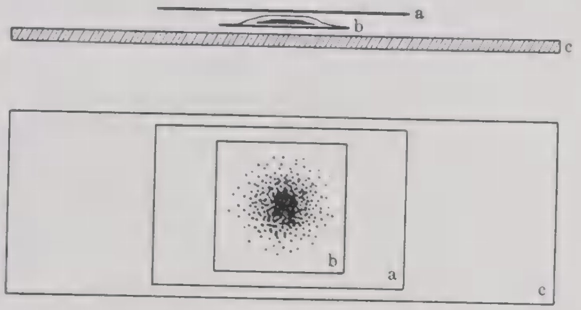

Fig. 72. Diagrams illustrating the procedure followed in mounting a stained culture grown on a mica coverslip: (*a*) glass coverslip; (*b*) mica coverslip (trimmed) with culture on upper surface; (*c*) glass slide.

In making whole preparations of flask and tube cultures in which the tissues are embedded in a coagulum, both the fixing and staining may be done *in situ*. Otherwise the coagulum may be removed from its container after preliminary fixation, but before it has completely hardened (see p. 247). When flask cultures are stained *in situ*, the flasks are kept with the culture side up, so that any precipitate formed will not settle on the cells. Fluids that do not pour readily may be withdrawn with a pipette; or the surface tension of the fluid may be altered by adding a drop or two of alcohol or acetone. Under no circumstances should the flasks be shaken. Any washing may be done at the tap by leading glass-tipped rubber tubing directly from the water line into the neck of the flask. The flow should be extremely moderate. Eventually the flasks are filled either with xylol or with oil of cedarwood, Florida extra, and fitted with corks. The corks are inserted very loosely in order to ensure against breakage resulting from expansion of the fluid contents with changes in temperature. For the same reason, a small air space is left just beneath the cork. Rubber stoppers are never used for this purpose.

Greater care must be taken in the preparation of fluid cultures in which the cells are growing directly on the glass. Under these conditions cells are extremely susceptible to violent treatment. As a rule it is desirable to flush flask cultures out with two or three changes of warm Ringer-formol, and to leave the flasks inverted and filled with the fixative, in the incubator, so that any débris will fall away from the cells. Subsequent washing is done with a pipette rather than with running water from the tap. Tube cultures are fixed and stained in an upright position. To retard the fading of delicate stains (such as Giemsa), the flasks are finally filled with oil of cedarwood, Florida extra, instead of with xylol. Oil of cedarwood of the immersion type hardens on exposure to air, whereas oil of cedarwood, Florida extra, remains fluid.

In the preparation of flask cultures for embedding and sectioning, extreme care is taken to avoid mutilating the cells. Cultures grown in a plasma or fibrinogen clot that is unusually thin should have a fresh coagulum superimposed on the first a few hours before the cultures are fixed. As already indicated, the cultures may be partially fixed before they are removed from the flasks (see p. 247). Otherwise they may be removed from the flasks before fixation (see p. 159). The excess plasma surrounding the culture is cut away with a sharp knife and the parts to be preserved are reembedded in a plasma coagulum to facilitate handling. The new plasma coagulum is prepared on a sheet of mica. (Mica is preferable to glass since it is nonbreakable and easy to mark for identification.) Before the culture is transferred to the new clot, the plasma is allowed to coagulate just enough to render it fairly firm. The trimmed culture is then spread out on the surface of the clot, where it is kept flat and protected from below by a layer of plasma. Once the culture has been oriented, a layer of plasma is spread over its surface and this in turn is firmly coagulated with tissue extract. After five or ten minutes, the entire coverslip is dropped into the fixing fluid. The subsequent treatment is the same as for coverslip cultures that are to be sectioned (see p. 250).

In making whole mounts of coverslip preparations in which the living material is embedded in coagulated plasma, it is desirable to rinse the cultures in several changes of warm Tyrode's solution or its equivalent, before fixation. The whole process may be carried out conveniently in small Coplin jars (Columbia staining dishes) in which the cultures can be rinsed, fixed and stained while held in a vertical position (Fig. 41). According to Murray,* the staining is improved if the preparations are stored immediately after fixation for several days, or even weeks, in 80 per cent alcohol.

During staining and washing, the introduction of metal objects into the solutions often injures the stain. Forceps used in staining should be provided with plastic tips.

Fixing Reagents

Ringer-formol

	Parts
Ringer's solution	9
Neutral formalin (40 per cent solution)	1

Formaldehyde is a gas and is dispensed as a 40 per cent aqueous solution known as formaldehyde U.S.P., formalin or formol. Four per cent formaldehyde (10 per cent formalin or formol) is the strength ordinarily recommended for fixation. It is prepared by diluting commercial formalin 1:9 with saline. Before it is diluted, the commercial material is neutralized by shaking with powdered calcium or magnesium carbonate.

Cultures embedded in plasma clots may be fixed with two or three changes of *Ringer-formol* (at incubator temperature) for at least one hour. If necessary, this particular fixing reagent may be allowed to remain on the culture for a much longer period, even for days. After fixation the cultures are washed thoroughly in running water, then in several changes of distilled water. Washing at the tap for from thirty minutes to one hour is usually sufficient for the average clot. If the cells are cultivated directly on glass in a fluid medium, the period of fixation may be reduced to ten minutes. Where the fixing fluid may wash away too many of the cells, the material is treated with *formalin vapor*. Thereafter the treatment is the same as with Ringer-formol except that washing at the tap is replaced by changing the water frequently with a pipette.

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* Murray, M. R. Personal communication.

Bouin's Fluid

	Parts
Picric acid (saturated aqueous solution)	15
Neutral formalin (40 per cent solution)	5
Glacial acetic acid	1

Bouin's fluid keeps well, has considerable penetrating power, and can be followed by almost any stain. It is suitable for either whole mounts or sectioned material. Because picric acid serves as a desirable mordant, it need not be completely washed away. Coverslip cultures may be fixed for one-half hour, flask cultures with a dense coagulum for a somewhat longer period. The preparations are then passed through several changes of 80 per cent alcohol, and are stored in this reagent until they are stained. Prolonged seasoning (several days to several weeks) in 80 per cent alcohol improves their staining qualities.

Zenker's Fluid

			Parts
Stock solution	Potassium bichromate	25 gm.	19
	Bichloride of mercury (crystals)	40 gm.	
	Water	1000 ml.	
Glacial acetic acid			1

(The first-named substances are brought into solution by heating. The glacial acetic acid is added just before use.)

Zenker-formol (Helly's Fluid)

	Parts
Zenker's stock solution	18
Neutral formalin (40 per cent solution)	2

(The formalin is added just before use.)

Zenker's fluid and *Zenker-formol (Helly's fluid)* should not be used for total preparations. These reagents leave a large amount of precipitate on the surface of the clot; they also render it granular and opaque. They may be used, however, when the cultures are subsequently to be embedded and sectioned. For this purpose, Zenker-formol is preferable to ordinary Zenker's fluid. Depending upon the size and thickness of the culture, the time required for fixation in Zenker-formol will vary from thirty minutes to two hours, or longer. After fixation, the cultures are washed for from one to six hours in running water and then dehydrated in the usual manner. A few drops of iodine are added to the 70 per cent alcohol to remove any bichloride of mercury that may remain in the tissues. If this is not done, crystals may form in the mounted sections. Earle recommends that cultures to be fixed in Zenker-formol should be first washed with warm formol-dichromate solution (see p. 246). The formol-dichromate is then replaced with Zenker-formol.

HEMATOXYLIN (HARRIS) STAIN FOR WHOLE CULTURES

1. After the cultures have been fixed in Helly's, Zenker's or Bouin's fluid, and washed, fill the flasks with a very dilute solution of Harris's alum hematoxylin (2 to 3 drops in 10 ml. distilled water) and let them stand overnight. If coverslip preparations are being stained, the hematoxylin may be placed in small Coplin jars (Columbia staining dishes). Care should be

taken to stain progressively to the desired degree and not to overstain. With a thick clot, the process of removing the stain is not only difficult to control but usually renders the color unstable. The stain may be removed, if necessary, with 0.5 per cent acid alcohol; the culture is then washed thoroughly at the tap. The blue color of the hematoxylin may also be restored by treating the culture with very dilute ammonia water (1 drop of the strong commercial solution in about 50 ml. of water). This treatment is again followed by a thorough washing at the tap.

2. Wash thoroughly in running water until both the culture and the coagulum have become bright blue in color. This washing may take from thirty minutes to one hour, or longer.

3. When an eosin counterstain is used, rinse the culture thoroughly in distilled water, then place in a 0.5 per cent solution of aqueous eosin for from five to ten minutes according to the intensity desired. After formalin fixation, tissues do not stain readily with eosin, hence a slightly higher concentration or a longer staining period is required.

4. Dehydrate with 95 per cent and absolute alcohols. Each alcohol is left on the culture until the clot has become thoroughly penetrated (that is, until convection currents are no longer discernible). If eosin has been used, the steps should be made as rapidly as possible; otherwise the stain will be entirely removed.

5. Clear in the following acetone-xylol mixtures:

Acetone, 95 per cent; xylol, 5 per cent
Acetone, 70 per cent; xylol, 30 per cent
Acetone, 30 per cent; xylol, 70 per cent
Pure xylol (two or three changes)

Let each mixture penetrate the coagulum thoroughly. If the alcohol steps have been abbreviated due to the presence of eosin, a prolonged treatment with the acetone-xylol mixtures will be necessary to insure both complete dehydration and adequate clearing. If the culture is of the coverslip variety, it may now be mounted; if it is contained in a flask, it remains only to fill the flask with oil of cedarwood and close the opening with a loose-fitting cork.

Harris's Alum Hematoxylin

Hematoxylin crystals	2 gm.
95 per cent alcohol	20 ml.
(Dissolve the hematoxylin in the alcohol)	
Ammonium alum	45 gm.
Distilled water	400 ml.

Dissolve the alum in the water by heating and then add the hematoxylin solution. Place the mixture in a 2-liter flask and bring it rapidly to a boil. To this add 1 gm. of mercuric oxide. The solution will boil very violently and will at once assume a dark purple color. Cool immediately by plunging the flask into a basin of cold water. After cooling, filter and keep in a well-stoppered bottle. This solution, which is now ready for use, will keep for several months. Although the addition of glacial acetic acid up to about 4 per cent increases the precision of the nuclear staining, it renders the solution unstable.

Acid Alcohol

	ml.
70 per cent alcohol	100
Hydrochloric acid, concentrated	5

MURRAY'S MODIFICATION OF MASSON'S TRICHROME STAIN FOR WHOLE COVERSLIP PREPARATIONS*

1. Fix in Bouin's or Zenker's fluid.

2. Treat Zenker-fixed cultures with a weak solution of iodine (e.g., 1:15 or 1:20 dilution of tincture of iodine) for five minutes, rinse with water, place for a few minutes in a 5 per cent solution of sodium thiosulfate and wash five to ten minutes in running water.

3. Store as long as possible (several days to several weeks) in

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* Murray, M. R. Personal communication.

80 per cent alcohol. Before staining, rinse quickly in distilled water.

4. Stain one-half hour in acid fuchsin-ponceau mixture (0.9 gm. ponceau de xylidine,* 0.1 gm. acid fuchsin, 1.0 ml. glacial acetic acid, 100 ml. distilled water; filtered) and rinse quickly in distilled water.

5. Differentiate in 1 per cent aqueous phosphomolybdic acid, fifteen minutes to one hour.

6. Drain and stain one minute in 1/2 to 1/3 strength anilin blue (2.5 per cent anilin blue in 2.5 per cent glacial acetic acid), and rinse quickly in distilled water.

7. Return to phosphomolybdic acid for five to ten minutes.

8. Fix for ten minutes in 1 per cent acetic acid.

9. Rinse quickly in 95 per cent alcohol and dehydrate in absolute alcohol.

10. Clear in methyl salicylate and several changes of toluene (water-free).

11. Mount in balsam or clarite.

Results: Nuclei and cytoplasm, red; collagen, and bone and cartilage matrix, blue.

Earle's Ferrous-Ferric Hematoxylin Procedure for Whole Cultures

Earle[78] has described a somewhat more elaborate procedure for making whole mounts of cultures from Carrel flasks. The method involves fixation in formol-dichromate (10 per cent formalin made up to 100 ml. with 3 per cent potassium dichromate) of controlled *p*H, hardening in 3 per cent potassium dichromate alone, staining in a ferrous-ferric hematoxylin solution, dehydrating in alcohols, acetone and toluene, and mounting in clarite.

In brief outline, the procedure is as follows:

1. Flask cultures to be stained are removed from the incubator, drained immediately, filled with fixing solution previously

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* Ponceau 2R, Colour Index Number 79, of the British Society of Dyers and Colourists.

warmed to incubator temperature, and returned at once to the incubator for ten to fifteen minutes to allow the fixing solution to penetrate the clot. The cultures are then removed from the incubator and the fixing solution is renewed after periods of ten, fifteen, fifteen, thirty and sixty minutes, and six, twenty-four and forty-eight hours, respectively. The frequent changes of the formol-dichromate solution serves to bring the *p*H to a satisfactory level for fixation by washing out the alkaline buffer of the culture medium.

2. After fixation, the culture flasks are rinsed and filled with tap water, and the plasma clots containing the cultures are removed from the flasks with a sharp, steel spatula (Fig. 21*C*). The preparations are hardened for five to eight days by placing them in a 3 per cent solution of potassium dichromate (at 4° C.), which is changed at any sign of discoloration.

3. After hardening, the preparations are washed in running tap water for twenty-four hours. If they are to be stored for a time, they are run up through increasing concentrations of alcohol, to 80 per cent. If they are to be stained immediately, they are transferred directly from water to a mixture consisting of 2 parts water, 1 part alcohol and 1 part glycerine; they must not be allowed to float in this mixture. They are then stained for two hours in Earle's special iron hematoxylin,* after which they are washed repeatedly and for a half-hour longer than is required to render the washings completely colorless (but not overnight). The coagulum is then trimmed of all extraneous plasma, except that several millimeters of cell-free clot are left at the periphery of the culture.

* Earle's[78] iron hematoxylin is made up as follows: *Solution A*, 0.9 gm. hematoxylin and 25 ml. absolute alcohol; *solution B*, 15 gm. ferric ammonium sulfate, crystalline reagent, and 50 ml. distilled water; *solution C*, 15 gm. ferrous sulfate, crystalline reagent, and 50 ml. distilled water; *solution D*, 25 ml. absolute alcohol and 50 ml. glycerine.

These solutions are freshly prepared. When ready for use, *A* and *B* are mixed with shaking and allowed to stand in an open flask for two hours. *Solution C* is then added and mixed. Thirty minutes later, *solution D* is added; and the entire solution is mixed in a glass-stoppered bottle and kept overnight. When kept at room temperature, this solution is good for thirty to sixty days, or until it begins to give the fibrin matrix of the culture a diffuse, brownish stain. Each time after use it may be filtered and returned to the stock bottle.

4. The stained cultures are dehydrated by passing them through 35, 50, 65, 80, 95 per cent and absolute alcohol and are then passed through six clearing mixtures (ten minutes in each) as follows:

Solution designation	1	2	3	4	5	6
			Parts			
(a) Absolute alcohol	70	50	25	0	0	0
(b) Acetone	25	25	25	25	10	0
(c) Toluene (water-free)	5	25	50	75	90	100

5. The cultures are then placed in clarite* solution and are finally mounted in an excess of clarite, which is held in place around the culture by means of a rectangular paper gasket cut from a single thickness of standard library card and laid flat on the slide. One end of the paper gasket is left partially open; and after a coverslip has been laid over the preparation, an additional drop of clarite is placed at the edge of the coverslip at the aperture of the chamber that is formed. The preparation is then placed in an incubator to dry. As the clarite hardens, it loses solvent and shrinks so that additional clarite is drawn in through the aperture in the paper gasket. The slides are examined daily and more clarite is added as needed. After four days lead weights are placed on the coverslip. If lead weights are added sooner, they may squeeze the paper gaskets out from under the coverslip. In about two months, the preparations are dry enough to clean and store (flat).

HEMATOXYLIN PROCEDURE FOR TISSUES CULTIVATED DIRECTLY ON GLASS IN ROLLER TUBES

1. Before fixation, wash cultures in several changes of warm Tyrode's solution, or its equivalent.
2. Fix overnight in Ringer-formol.
3. Rinse several times with distilled water.

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* 200 gm. of clarite (Neville Co., Pittsburgh) dissolved in 100 ml. of toluene.

4. Stain three to five days in full strength Delafield's alum hematoxylin. Overstained cultures may be destained in acid alcohol (see p. 245). Understained cultures may be rinsed with distilled water and stained again.

5. Wash with dilute ammonia water (see p. 244) until the tissues have become deep blue in color.

6. Wash in several changes of distilled water.

7. Fill culture tubes with glycerine and close with rubber stoppers.

Delafield's alum hematoxylin: To 400 ml. saturated aqueous ammonium alum, add 4 gm. hematoxylin dissolved in 25 ml. 95 per cent alcohol. Leave exposed to air and light for four days. Add 100 ml. methyl alcohol, 10 ml. glycerine, and filter. The filtrate should ripen for six to eight weeks.

Giemsa Stain for Leucocytes Cultivated in Flasks in Fluid Media

1. Withdraw the culture fluid and rinse the flask with warm glucosol solution (p. 79). Keep the flask turned over as much as possible so that the cells are on the top side and will not be disturbed by the introduction of fluids. If fluids do not pour off readily, remove them with a pipette.

2. With the flask still inverted, introduce about 1 ml. of warm 10 per cent Ringer-formol. Do not allow the fixative to come into contact with the cells. Stopper and place in the incubator for ten minutes or longer.

3. Pour off the Ringer-formol and rinse with distilled water until the odor of formalin is no longer perceptible.

4. Introduce Giemsa stain in the dilution of 1 drop of stain to 2 ml. or more of distilled water, depending upon the nature of the stain. Some stains may be left on the cultures overnight, others require only an hour or so. In any event, renew the stain after the first hour. The flask is filled completely, loosely stoppered and inverted to prevent any precipitate from settling on the cells.

5. Rinse twice, quickly but thoroughly, in each of the following fluids:

Acetone, 2 parts; xylol, 1 part
Acetone, 1 part; xylol, 2 parts
Pure xylol.

6. After the final xylol, fill the flask with oil of cedarwood, Florida extra, and close loosely with a cork.

Note: Unfortunately, this stain is not permanent.

TECHNIQUE OF SECTIONING CULTURES

1. As already described on page 240, flask cultures to be sectioned are removed from the flasks in the usual manner and re-embedded in a fresh plasma coagulum on sheets of mica. If this is not done, the cultures will shrink and curl to such an extent that it will be impossible to obtain flat sections. The additional coagulum serves also to protect the culture from damage in subsequent handling. Plasma cultures carried as hanging drops can be fixed directly. It is sometimes desirable, however, to give them the protection of an additional, superficial layer of plasma.

2. Immerse the mounted culture in the fixing fluid. With Zenker-formol the time required for fixation will vary from thirty minutes to two hours, or longer, according to the size and thickness of the culture.

3. Wash for from one to six hours in running water, unless the particular type of fixation requires some other form of treatment.

4. Dehydrate and harden:
   (a) Fifty per cent alcohol, thirty minutes, or longer.
   (b) Seventy per cent alcohol, one hour to overnight. If a fixative containing bichloride of mercury has been used, the cultures should be left overnight in a dilute solution of tincture of iodine in 70 per cent alcohol, followed by several changes of fresh 70 per cent alcohol. If it is necessary for the cultures to stand for a consid-

erable period of time before embedding, e.g., over a week-end, they may be left in 70 per cent alcohol.

(*c*) Eighty per cent alcohol, one hour.

(*d*) Ninety-five per cent alcohol, one hour to overnight.

(*e*) Absolute alcohol containing a few drops of a strong solution of alcoholic eosin in absolute alcohol, two changes, one to three hours each. The eosin colors the material in such a manner that the culture may still be seen after it has been embedded in paraffin.

(*f*) Transfer to fresh absolute alcohol for from twenty to thirty minutes.

5. Clear as follows:

(*a*) Clear the culture with two per cent parlodion in methyl benzoate (two changes) until both culture and clot are perfectly clear and transparent. If a culture of the average size does not clear in one hour, or less, return it to absolute alcohol for further dehydration.

(*b*) Place in two changes of benzene. In the first of these, the culture is cut away from the mica with a thin, sharp knife or razor blade. Begin by making four vertical incisions through the clot at the line of demarcation between the old and the new coagulum. Then, holding the knife blade almost parallel to the culture, separate the clot from the mica by a gentle cutting action, exerting any necessary pressure on the mica. Care must be taken, however, not to cut into the latter, for any mica chips that remain attached to the culture may nick the microtome knife. During the process of detaching the culture, both the knife and the coagulum are kept well moistened with benzene. The total benzene treatment takes from five to fifteen minutes, or longer. Once the culture has been removed from the mica, it should be handled with a section lifter, never with forceps.

6. Impregnate and embed:

(*a*) Benzene-paraffin mixture at 40° C., five to ten minutes, or longer;

(*b*) Paraffin, melting point 50 to 52° C., five to ten minutes, or longer;

(*c*) Paraffin, melting point 56 to 58° C., five to ten minutes, or longer;

(*d*) Embed in fresh paraffin (56 to 58° C.). When the paraffin has cooled, the embedded culture is ready for sectioning.

7. The paraffin block is trimmed in such a manner that flat sections of the entire culture may be obtained. With a sharp microtome knife, serial sections (5 to 7 microns) of the whole culture are cut. As the ribbon comes away from the knife, it is placed in proper sequence in a shallow box on a sheet of smooth, unglazed paper. The clot, tinted by the eosin, is readily distinguished from the surrounding paraffin. In addition, with the aid of a strong magnifying glass, sections containing tissue may often be distinguished from those containing clot only. The tissue stains a brighter red than the clot.

8. The paraffin ribbons are now mounted on glass slides that are flooded with distilled water containing 1 drop of Mayer's egg albumen mixture to about 75 ml. of water. After the slides have been heated gently on a hot-plate, the ribbons are stretched and flattened with fine needles. Care must be taken to avoid heating the hot-plate above the melting point of the paraffin, for under no condition should the ribbon be melted. Then, without wiping away the excess water, place the flattened sections in an incubator to dry at 40° C. When thoroughly dry, the ribbons have a translucent appearance, and are ready for staining.

Tincture of Iodine Solution

Potassium iodide	5 gm.
Iodine crystals	7 gm.
Distilled water	10 ml.
95 per cent alcohol, to make	100 ml.

Dissolve the potassium iodide and the iodine crystals in the water by allowing them to stand overnight in a well-stoppered bottle. On the following day, add the alcohol.

Parlodion-Methyl-Benzoate Solution

Parlodion	2 gm.
Methyl-benzoate (pure), about	100 ml.

Parlodion is the trade name given to celloidin strips. The strips are washed with distilled water and dried. They are not readily soluble in the methyl-benzoate; hence, the solution is prepared several days in advance. It is stable and may be used repeatedly.

Benzene-Paraffin Mixture

	Parts
Benzene	1
Paraffin (50 to 52° C.), melted	1

This mixture solidifies at room temperature, but remains fluid at 40° C.

Mayer's Egg Albumen Mixture

	Parts
Fresh egg albumen	1
Glycerine, pure	1

Beat together and filter through paper. Preserve with a crystal of thymol or with 1 per cent sodium salicylate. The mixture keeps for many months.

General Embedding Procedure for Control Tissues and Necropsy Material

1. Fix the tissue in Zenker's fluid or in Zenker-formol, six hours to overnight.
2. Wash in running water overnight.
3. Fifty per cent alcohol, one hour.
4. Seventy per cent alcohol with a few drops of tincture of iodine, at least six hours.
5. Seventy per cent alcohol (here, tissue may be left indefinitely).

6. Eighty per cent alcohol, one hour.

7. Ninety-five per cent alcohol, overnight.

8. Absolute alcohol, two changes, two hours each.

9. Absolute alcohol-benzene (1:1) mixture, thirty minutes to one hour.

10. Benzene, two changes, thirty minutes to one hour each.

11. Benzene-paraffin (1:1) mixture, thirty minutes to one hour.

12. Paraffin (56 to 58° C.), two changes of thirty minutes each.

13. Embed in paraffin.

Note: Steps Nos. 8 to 13, inclusive, are so arranged as to complete the procedure in one working day.

Hematoxylin-Eosin Procedure for Sections

1. After removing the paraffin from the mounted sections, with xylol, toluol or benzene, place them in absolute alcohol and then run them down through the graded alcohols to distilled water.

2. Stain with Harris's hematoxylin, one to fifteen minutes, or longer; overstain slightly.

3. Rinse thoroughly, first with distilled water and then with running tap water.

4. Differentiate with acid alcohol. Here the sections become pink.

5. Rinse quickly in running tap water and neutralize with a weak ammonia solution (1 drop in about 200 ml. of water). Here the sections become blue. If the ammonia solution is too strong, there is danger of detaching the sections from the slides.

6. Wash for at least fifteen minutes in running tap water.

7. Stain for three minutes with 0.1 per cent aqueous eosin.

8. Run the sections up rapidly through the graded alcohols, dehydrate with three changes of absolute alcohol, clear in three changes of xylol or toluene, and mount in Canada balsam or clarite.

Bodian's Silver Impregnation Method for Nerve Structures in Paraffin Sections

Bodian[25] has found that much of the selectivity of silver stains is dependent upon the initial fixation. The most generally satisfactory fixative is formol (one part), glacial acetic acid (one part) and 80 per cent alcohol (eighteen parts). Satisfactory results have also been obtained with Bouin's fluid. Fixatives containing chrome salts are not recommended. After fixation, paraffin sections are prepared in the usual manner (see p. 250). The subsequent procedures may be summarized as follows:

1. Remove paraffin with xylol and run sections through absolute alcohol and 95 per cent alcohol to distilled water.

2. Place sections in a solution of 1 per cent protargol (silver albumose) containing 4 to 6 gm. of metallic copper (e.g., copper wire) per 100 ml.; twelve to twenty-four hours at 37° C. (The protargol-copper bath can be used only once.) Wash in distilled water.

3. Reduce for ten minutes in the following solution:

Hydroquinone	1 gm.
Sodium sulfite	5 gm.
Distilled water	100 ml.

Wash in at least three changes of distilled water. If toning with gold is not desired, the sections are now dehydrated and mounted in balsam. For double impregnation (to accentuate certain structures that stain very lightly), the sections, after having been washed, are placed in a second bath of fresh protargol and copper and are subsequently reduced as before.

4. If greater contrast is desired, the washed sections may be toned in 1 per cent gold chloride containing three drops of glacial acetic acid per 100 ml. of solution, until they are decolorized (usually two to five minutes). Wash in distilled water.

5. Place sections in 2 per cent oxalic acid until they become slightly blue or purple (usually from two to five minutes). Wash in distilled water.

6. Remove residual silver salts in 5 per cent sodium thiosulfate (hypo), five to ten minutes.

7. Wash thoroughly in distilled water, dehydrate and mount in balsam or clarite.

Note: This method may be adapted, very simply, for use with whole cultures.

A Technique for Staining Bacteria in Cultures*

PROCEDURE FOR LEUCOCYTES IN FLUID MEDIA

1. Rinse culture flask with warm Ringer's or Tyrode's solution and fix with 10 per cent Ringer-formol for at least thirty minutes. Observe all the precautions that were indicated in connection with the use of Giemsa stain (p. 249).

2. Wash in running tap water for a few minutes.

3. Stain for one minute with carbol-aniline-fuchsin (see p. 257).

4. Wash in running water, one minute.

5. Decolorize for a few seconds with 1 per cent acetic acid in 40 per cent formaldehyde (commercial stock solution).

6. Wash in running water, one minute.

7. Counterstain with a saturated aqueous solution of picric acid, one minute.

8. Wash in running water, one minute, and repeat the entire procedure from Nos. 3 to 7 four or five times, according to the depth of coloration desired. When treating the cultures for the last time with picric acid, allow the acid to act for three minutes.

9. Dehydrate very rapidly in three changes of absolute alcohol. If this is not done quickly enough, the picric acid will be removed.

10. Clear with two changes of pure xylol. Fill the flask with a third change of xylol, or replace this third change of xylol with two changes of oil of cedarwood, Florida extra, and allow the latter to remain.

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* Adapted from Goodpasture, E. W. *Am. J. Path.*, 1925, *1*, 547.

PROCEDURE FOR SECTIONED CULTURES

1. Fix with Zenker-formol, dehydrate, clear and embed in the usual manner (see p. 250). Cut very thin sections, i.e., less than 5 microns in thickness.

2. Remove the paraffin with xylol and run the slides down through the graded alcohols to distilled water. The procedure is then the same as that indicated for leucocytes in fluid media, beginning with step No. 3.

3. Mount the sections in Canada balsam.

Carbol-Aniline-Fuchsin

	ml.
Carbolic acid or phenol (pure)	1
Aniline oil	1
Basic fuchsin, 2.5 per cent in alcohol	20
Distilled water	80

(This solution should be prepared fresh each time, and filtered before using.)

A Technique for Acid-Fast Bacteria in Cultures

The procedure to be described has given excellent results in connection with fluid cultures of leucocytes containing avian tubercle bacilli (Fig. 71). Cultures embedded in a plasma coagulum, or those in which the cell layer is very thick, must be sectioned. After sectioning, the staining procedure is the same as that for whole cultures, as follows:

1. Rinse culture flask with warm Ringer's or Tyrode's solution and fix with warm 10 per cent Ringer-formol for at least thirty minutes.

2. Wash in running water, fifteen to thirty minutes, or longer.

3. Rinse with five or six changes of distilled water and stain with Harris's hematoxylin (see p. 245). Use no acetic acid in the hematoxylin and overstain only very slightly, if at all.

4. Wash thoroughly, first in running water at the tap and then with several changes of distilled water.

5. Stain overnight with Ziehl-Neelson's carbol-fuchsin (see p. 258).

6. Rinse well in running water.

7. Decolorize very rapidly with 3 per cent nitric acid in 95 per cent alcohol. This should be accomplished in from twenty to thirty seconds, to prevent removing the hematoxylin.

8. Rinse well in running water and neutralize with a dilute solution of ammonia (1 drop of concentrated ammonia to 100 ml. of water) in order to restore the blue of the hematoxylin; then wash in running water for from five to ten minutes. The cytoplasm and nuclei should be blue, the bacteria, red. If the cells have retained the red of the fuchsin, they should be decolorized still further with the 3 per cent nitric acid in 95 per cent alcohol.

9. Dehydrate, clear and mount quickly as follows:
   (*a*) Ninety-five per cent alcohol, two changes.
   (*b*) Absolute alcohol, two changes.
   (*c*) Pure xylol, two changes.
   (*d*) Fill the flask with a third change of xylol, or replace this third change of xylol with two changes of oil of cedarwood, Florida extra, and allow the latter to remain.

Ziehl-Neelson's Carbol-Fuchsin

	ml.
Saturated alcoholic solution of basic fuchsin	10
5 per cent carbolic-acid water	90

(The carbolic-acid water is prepared by adding 95 ml. of hot water to 5 ml. of melted carbolic acid crystals [phenol]. It is then filtered.)

CHAPTER XVII

PHOTOMICROGRAPHY AND MICROCINEMATOGRAPHY

PHOTOMICROGRAPHY, which provides the only efficient means of recording the outward appearance of cells, is indispensable as an adjunct to the methods of tissue culture. If it is to serve its full purpose, however, the arrangements must be extremely simple, inexpensive to operate and the techniques so standardized that satisfactory negatives can be made at any moment by any member of the laboratory staff.

Microcinematography, or motion photomicrography, provides a means of recording the constant changes undergone by cells in function of time. With the microcinema, it is possible to study the particular manner in which different types of cells migrate and form colonies, the complex changes that accompany cell division, the nature and behavior of the mitochondria and the segregation apparatus, how cells take in fluids[167] and particulate substances, and how they react to physicochemical changes occurring naturally or produced artificially in their immediate environment. Some of these phenomena progress very slowly and would be missed entirely were it not possible to create the illusion of speeding them up by condensing hours and even days of activity into a few seconds or minutes of actual screen time. It is also possible with the microcinema to "repeat" phenomena that occur only once in nature; and much that is overlooked the first time may be discovered the second. Moreover, cinema records offer one of the best means of instruction. No course in cytology or histology is ever complete until the student has been given the genuine thrill of seeing living tissues in the process of development.

In the present chapter both types of photography will be discussed.

PHOTOMICROGRAPHY

It is hardly necessary to state that the following pages have not been prepared for the professional photographer, but for the investigator who is relatively inexperienced in photomicrography and has neither the time nor the opportunity to seek out the information for himself. The procedures recorded here have been tested thoroughly and found to be adequate. Countless other combinations of materials and methods would doubtless serve the same purpose. But if the present instructions are followed carefully and systematically, it should be possible for anyone, with a little practice, to make a satisfactory negative in less than ten minutes.

Apparatus

An elaborate apparatus is by no means a necessity. Very often the simplest sort of equipment will suffice. There are available, for instance, the small micro-attachment cameras that can be fitted directly to the tube of the microscope. These accommodate standard plates and cut film. There are also the larger and more conventional types of laboratory cameras with upright, adjustable bellows. Other combinations are special adaptations of the miniature roll-film cameras for taking a series of still pictures on ordinary 35-mm. cine film. But while all of these devices have, at one time or another, been found adequate for general photomicrography, and for work with hanging-drop preparations, they fall definitely short of providing the most ideal conditions for photographing living cells cultivated in flasks. This fault does not lie with the cameras themselves, but results from the necessity for using them with the common type of laboratory microscope. Thus, flask preparations that are placed on the stage of an ordinary microscope must be inverted in order to bring the objective close to the culture (Fig. 73*A*, *B*). Under these conditions, the fluid drains away from the cells, leaving them to be killed by evaporation. Although cultures are placed in just this position for brief periods of observation under an ordinary microscope, the light is usually less intense than that used for photography and the temperature approximates that of the room. Even at room temperature cells soon dry out in inverted flasks; and this evaporation occurs with surprising rapidity when the medium is entirely fluid, with no plasma coagulum.

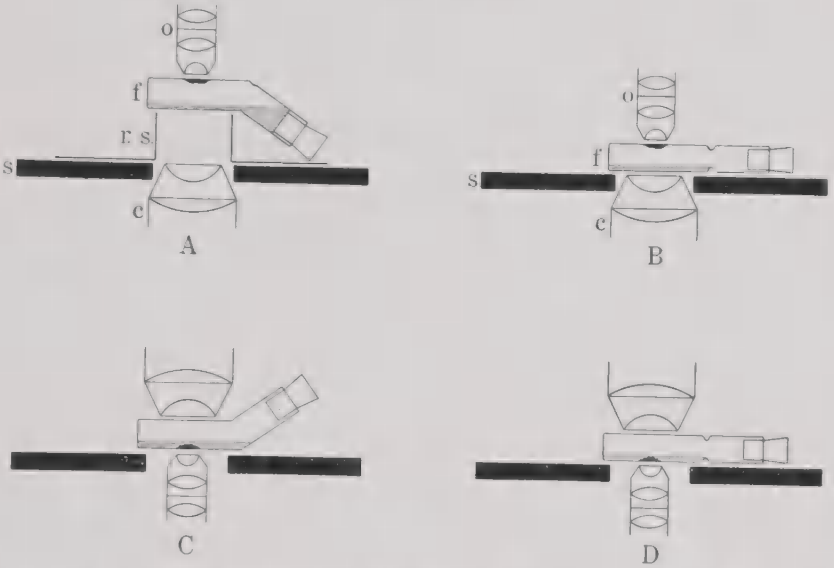

FIG. 73. Diagrams illustrating the advantages to be gained by using an inverted microscope when working with Carrel culture flasks. When cultures are photographed with an ordinary microscope, the flasks must be turned bottom side up (*A* and *B*), leaving the cells to dry out. An inverted microscope, with the light source above the stage and the objective beneath (*C* and *D*), makes it possible to work with the cultures without displacing the medium. (*o*) objective; (*f*) flask; (*c*) condenser; (*s*) stage; (*rs*) ring-stand support.

Another complication is caused by the layer of fluid that forms on the lower surface of an inverted flask (Fig. 73*A, B*). This accumulation offers an extra barrier to scatter and deflect the light rays destined to form the photographic image. Consequently, in order to overcome these difficulties, an arrangement is preferable whereby the microscope is inverted, with the light source and condenser above the stage, and the objective, eyepiece and camera beneath. Such a system renders it possible to place the flasks on the stage in their natural position, i.e., one in which the cells are protected and bathed at all times with the culture medium (Fig. 73*C*, *D*). In addition, the inverted microscope makes it relatively simple to photograph cells cultivated in the heavier flasks with the inclined necks (Fig. 73*C*). A special long working distance condenser (e.g., the Leitz bright field condenser: working distance, 15 mm.; N.A., 0.75) can be used to focus light rays through the chamber of these flasks upon the cells to be photographed. When an ordinary microscope is used, such a flask must be inverted and supported on a special stand in order to give a flat working field (Fig. 73*A*). With this inversion, illumination is adequate for routine visual observation, but the same light intensity is insufficient for photographic purposes.

The entire photographic apparatus should be rigid, heavy and so constructed that any vibration will affect the entire system rather than throw any one part out of alignment with respect to the others. In the ideal arrangement, the light source, condensing and cooling systems, filter holders, microscope, and camera are all attached to a well-constructed optical bench. Such a contrivance, combining all the features of the inverted microscope, the optical bench, and the camera attachment, already exists in the form of the Leitz "Edinger" apparatus. With this apparatus (fitted with Zeiss "Homal" or Bausch & Lomb "Ampliplan" eyepieces), and modest dark-room equipment, it is possible to produce 4×5, 5×7 or 8×10 in. photomicrographs at magnifications of more than 1000 diameters.

Lenses

The optical equipment generally required for low-power work comprises achromatic objectives and appropriate eyepieces for photographing general features. For high-power investigations, fluorite (semi-apochromatic) and apochromatic objectives are preferred owing to their better color correction and increased resolving power. Objectives like the Leitz fluorite oil immersion $\frac{1}{7}$a and the 3-mm. apochromatic oil immersion (N.A. 1.32) have been used very successfully. It is also desirable in high-power photography to use eyepieces of the Zeiss "Homal" or Bausch & Lomb "Ampliplan" type. Although these lenses are referred to as eyepieces, they are actually negative lenses that produce on the focusing screen, or photographic plate, a magnification of the intermediate image formed at the upper end of the microscope tube. Because they compensate for the curvature inherent in the intermediate image of any high-power objective, they produce a greater area of flatness in the projected image than is produced by the conventional type of eyepiece. They also compensate for Abbe's chromatic differences of magnification. Homals and Ampliplans cannot be used for visual observation; they are primarily intended for photomicrography and for projection. And since they do not fit the eyepiece sleeve of standard tubes, suitable adapters are required. These adapters also give the correct tube length and provide a light-tight joint between the microscope and the camera.

When photomicrographs are to be made at extremely low magnifications, *Micro-Summar* and *Micro-Tessar* objectives are valuable because with them it is possible to cover large fields with a greater depth of focus than with ordinary low-power objectives.

Light Sources

As pointed out by Zieler,[278] the requirements for satisfactory illuminations are as follows: (1) The area of illumination must be of definite diameter, according to the objective and eyepiece that are used. It varies from several millimeters for low magnifications to 0.1 to 0.2 mm. for the highest oil immersions. (2) The intensity of the illumination at the periphery must be equal to that in the center or in any other part of the field. (3) The intensity must be uniformly variable by regulation of the aperture of the illumination and must permit a maximum value where this aperture is at least equal to that of the objective. Any method of illumination that fulfills these three conditions is perfectly satisfactory for photomicrography.

For low-power work it is possible to use one of the various low-voltage microscope lamps. The Leitz "Monla" lamp, for example, is equipped with a 6-volt, 5-ampere bulb, the intensity of which can be regulated by means of an adjustable transformer or resistance. "Monla" lamp illumination is comparable to that obtained by using a point-o-lite lamp. The filaments of the bulb are very narrow and do not interfere with the uniformity of the illumination, especially when this is adjusted according to the method of Koehler.[80]

At high magnifications a more intense source of light is required for the shortest possible exposure and for the use of filters. Everything considered, the electric arc is the most satisfactory source of illumination in photographing living cultures. The intensity of arc lights is so great that only very short exposures are necessary even though heat and color filters are interposed. In the author's laboratory, a 100-watt Western Union concentrated-arc lamp is used. This lamp, which operates on direct current and has a life expectancy listed at 1000 hours, is provided with permanent, fixed electrodes that are sealed into a glass bulb filled with an inert gas. The highly intense light source is in the form of a luminous circular spot (1.5 mm. in diameter) that is fixed in position, sharply defined and uniformly brilliant.

Materials

In photographing living, unstained cultures grown in a colorless medium, Eastman Process Film[80] may be used. But when the culture medium consists of serum or plasma containing phenol red, the degree of contrast is so slight that the work is best done with filters. The use of filters necessitates color-sensitive materials. When the filters used transmit blue, green or yellow light, orthochromatic plates or films may be used, but when orange or red filters are required, it is necessary to use panchromatic materials. Panchromatic materials have the great advantage of being suited to any color filter. Furthermore, their speed is considerably greater than that of ordinary orthochromatic or process films. Cut films are to be recommended instead of plates. They are unbreakable, easier to store and less expensive. For general purposes, the 5×7 in. size has been found to be the most satisfactory. The individual film is held in the ordinary plate holder by means of a metal film sheath.

When panchromatic materials are used, they should be developed in complete darkness; otherwise, by the light of a Series 3 Wratten Safelight (green) kept well apart from the covered tank or tray container. Panchromatic materials are sensitive, of course, to all colors, but green has been chosen for the Series 3 Safelight because it is the one color by which the eye can see the most with the least possible light.

Contrast

The greatest difficulty in photographing living, unstained cultures consists in obtaining sufficient contrast to give a good print. Furthermore, the contrast must be very real and appreciable, if it is to survive the engraver's laboratory at the time of reproduction. Sometimes, insufficient contrast is produced by unsatisfactory lighting. If this is suspected, the apparatus should be checked and centered, and its lighting system carefully re-aligned.[278] At other times, insufficient contrast may be caused by too large an aperture in the condenser diaphragm. But although the diaphragm should be closed down until all scattered light disappears, beyond a certain point this procedure spoils rather than improves the definition. With apochromatic objectives, resolution is best with a relatively large opening.

With the ordinary microscope the main control of contrast rests in the selection of filters. When unstained material is illuminated by an arc lamp and is photographed with apochromatic objectives and panchromatic film, a red filter or a combination of yellow and red may be used. But for preparations stained either supravitally or with permanent dyes, the investigator should select the filters by making, through them, a visual inspection of the area to be photographed. Care must always be taken, of course, not to sacrifice detail for contrast. Greatly increased contrast may be achieved by means of *dark-field* and *phase-contrast*[16, 94] microscopy. The latter is particularly well suited for work with living, unstained cultures mounted on perforated slides (see p. 144).

Exposure

In the beginning, correct exposure times may be reached empirically by making a series of trial exposures[80] on a single plate or film. Fortunately, however, some of the many factors determining the time of correct exposure remain relatively constant for each apparatus. Hence, if full tabular notes of past experiences are kept, the proper exposure time may usually be determined without the sacrifice of a plate.

Developing and Fixing

Panchromatic materials, which cannot be illuminated enough for inspection, are developed for a specified period of time, at a definite temperature. The developing time is determined by the nature and strength of the developer; the temperature, within certain limits, governs somewhat the degree of contrast obtained in the negative. The standard temperature generally adopted for negative development is 65° F. (18° C.). Higher temperatures may cause a greater amount of fog for a given image density. The actual developing solutions and the processing procedures should be those recommended by the manufacturer of the plates and films that are used.[80]

A plate or film to be developed in a tank is first inserted in one of the specially devised hangers or racks. This rack is then dropped into the developing solution and kept in motion throughout development. Where the amount of work is not too great, however, the negative is developed in a tray and transferred to the rack as soon as it is removed from the developing solution. If the racks are numbered, appropriate notes can be kept to prevent confusion.

A negative to be developed in a tray is immersed quickly and evenly if one first tips the tray, then drops in the negative, and causes the developer to flow back over it by lowering the raised side. The tray is then covered and the materials are agitated at frequent intervals during development. Also, when several negatives are developed together, they are placed in the developer one at a time and removed, eventually, in the same order. The only satisfactory timing device for a dark-room is an interval timer.

Before the negative is transferred to the fixing tank, it is well rinsed in a tank or tray of clean water. Negatives are allowed to fix for at least twice the time necessary to clear the film of all milkiness, and they are agitated frequently. At least ten minutes are required if the fixing solution is reasonably fresh. The bath is discarded when the total clearing time has about doubled that required in a fresh bath. Efficient fixing and hardening solutions are recommended by the manufacturers of the plates and films that are used.[80]

After the negatives have been thoroughly fixed they are washed for at least thirty minutes in a tank of running water; they are then wiped carefully with a soft sponge or chamois, hung up in their racks to dry, and later transferred to properly inscribed manila or glycine envelopes (without center seams). Finally, the negative number, the experiment number and the magnification are written in black ink on the reverse (shiny) side of each negative. When the prints are made, these notations will be transferred automatically to the paper.

Printing

There should be no difficulty in obtaining satisfactory prints from a good negative. For purposes of reproduction the printing paper that is selected should have a glossy surface and be of single weight. Besides, it should be available in several degrees of contrast. The grade of paper designed for excessively thin or flat negatives will probably be used almost exclusively in making prints of negatives from living, unstained preparations.

For the average laboratory, a special printing device is unnecessary. The paper and negative may be placed in an ordinary printing frame and exposed under a goose-neck table lamp. If the printing frame is of the same size as the negative, a white border will be obtained automatically without the use of a special mask.

After the prints have been developed and fixed (according to the manufacturer's recommendations for the papers that are chosen), they are placed for five minutes in a fresh fixing bath and then washed for two hours in running water. When the prints have been thoroughly washed they are removed from the wash water, drained and placed on chromium ferrotype plates for drying. After the excess water has been taken up with blotters or paper toweling the prints are thoroughly flattened with a rubber squeegee. If the humidity is low the prints should be placed in a bath of 10 per cent glycerine for thirty minutes before being rolled out on the ferrotype plates. This procedure will prevent subsequent curling and cracking of the glossy emulsion.

MICROCINEMATOGRAPHY

Microcinematography, or motion photomicrography, was first applied to the study of tissue cultures by Braus.[27] This investigator photographed pigment cells in hanging-drop preparations at the rate of 14 exposures per second; and, in 1911, he showed his early films at Karlsruhe. Later work of this nature was done by Comandon, Levaditi and Mutermilch,[61] and by Fabbri and Ebeling. In more recent years, Carrel, Ebeling and Rosenberger,[41, 47, 228] Canti,[31] Lewis,[164, 168] Fischer,[97] Gey,[94] and others, have made systematic use of the microcinema in studying some of the more fundamental differences between the various cell types. Ordinarily, from three to six exposures are taken per minute.

Cultures intended for microcinematography may be prepared in the thin-walled microflasks (see p. 46), on glass coverslips attached to ordinary thin slides by means of metal (or glass) rings (see p. 143), or in glass coverslips sealed to perforated slides (see p. 144). It is impossible, of course, to make successful photographs of coverslip preparations attached to heavy depression slides. But cultures that are originally prepared on depression slides may be transferred to ring slides or to perforated slides before they are photographed. For two or three-day cinema records at relatively low magnifications, the microflask is recommended. The length of time that cultures may be left undisturbed in microflasks depends solely upon the nature of the tissue and the frequency with which the medium must be renewed; but this period is considerably longer than the period during which a coverslip culture may be studied, particularly if the air space has been eliminated by sealing it to a perforated slide.

For culture work, a microcinema apparatus must fulfill two very necessary requirements if the project is to be successful. First, the microscope should be inverted in the manner described on page 261; otherwise, and for the reasons stated there, it will not be possible to work with flasks. Secondly, the entire apparatus should be enclosed in a constant-temperature room or cubicle[277] if preparations are to be kept in focus at high magnifications for long periods (such as overnight). It is not sufficient to place only the microscope in an incubator. The camera, its metal support, and the microscope stand should also be included, for with very slight variations in temperature, they will all contract and expand enough to throw the preparation out of focus. The actual temperature of the room is unimportant, but it should not fluctuate more than one or two degrees and should not be too far below that of the incubator in which the culture is kept.

Loveland,[179] who has made an extensive study of the conditions required for microcinematography of biological material, makes the following recommendations in regard to the apparatus:

1. If a vibration-free floor is available, the microscope should be firmly fastened to a solid, stable support.

2. The microscope should be of heavy construction and mechanically well built if it is to stay in focus over a period of many hours and give a vibration-free image. Commercial microscopes vary greatly in this respect.

3. The camera support should be independent of the microscope support, very stable, and uninfluenced by the intermittent camera motion. It should be massive with respect to the mass of the camera or should be firmly fixed to such a mass.

4. If the total mass is sufficient, the motor drive unit may be mounted on a floating system (sponge rubber, for example),

with a double universal joint connection. A magnetically operated clutch placed between the motor drive and the camera is useful when operating at normal or higher speeds.

5. The camera should run smoothly and easily, with small inertia in the moving system and, unless it is supported on its face, should have a broad base and low gate position.

6. It should be possible to raise or lower the camera vertically (or in the direction of the axis of the microscope) at least enough to free it from the light lock and to accommodate the different positions of the microscope tube required by different optical systems.

7. It should be possible to swing the camera out of the way for visual work and yet have it instantly ready and in alignment when needed.

8. It is advantageous to be able to see the field while it is being photographed. For this purpose, some sort of split-beam observation device[80] is needed. The visible area should be considerably larger than the actual camera field so that selections can be made while the equipment is in operation.

9. A pair of cross hairs should be placed in the plane of the correct visual focus as well as lines to indicate the limitations of the camera field; and it should be possible to focus the eye lens of the observation device separately on them. These arrangements will make it possible for different observers to obtain the same degree of critical focus in the area being photographed.

10. It is advantageous to have a picture of a watch in one corner of the film so that time-lapse may be indicated quite independently of the speed of projection. This requires an independent lens and a specially modified camera gate; the prism or mirror can serve as a mask.

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INDEX

Acid alcohol, 245
Acid-fast bacteria in cultures, staining of, 257
Amino acids, 116, 122, 127, 129, 231, 232
Ammonia solution, 244
Anaesthesia for chickens, 94
Antibody formation, 32, 205
Anticoagulants, 98
Antimetabolites, 115
Antitryptic agents, 173
Aorta, bleeding from, 99
Areas, surface, measurement of, 218
Ascitic fluid, 2
Assay procedures:
  with fibroblast strains, 163, 164, 165
  with fresh tissues, 175, 210, 220
  with leucocytes, 166, 180, 187
Assistance, technical, 54

Bacteria in cultures, staining of, 256
Balanced salt solutions, 76
Benzene-paraffin mixture, 253
Biological test material, 10, 109, 158, 163, 164, 165, 166, 175, 180, 183, 187, 210
Blastoderms, chick, 13
Bleeding, for plasma and serum, 88
Blood leucocytes, flask methods for, 166, 180
Blood vessels, suturing, 101
Bone formation, 11, 13, 109
Bouin's fluid, 242
Brushes, nail, sterilization of, 65
Buffers, phosphate (Sörensen), 83
Buffy-coat cultures, preparation of, 166, 180

Cameras, 260, 269
Carbol-aniline-fuchsin, 257
Carbon dioxide for adjusting *p*H, 153
Carcinogenesis, 18, 22
Carcinoma, 16, 18, 22, 26, 163, 166
Carotid artery, bleeding from, 91, 101
Cartilage, development of, 13
Cell migration, 18, 108, 219, 222, 223, 228
Cell multiplication, 10, 18, 108, 187, 219, 223, 234
Cell nutrition, 18, 107, 115
Cell types, 8, 9, 10
Cellophane, perforated, for fluid cultures, 26, 188, 195
Cells, single, isolation of, 26, 160
Cellular adaptation, 19, 26
Cement, waterproof, for culture containers, 198
Centrifuge tube (Lindbergh) for preparation of serum, 103
Chemically-defined media, development of, 115
Chlorazene solution, 71
Citrate, sodium, as anticoagulant, 99
Clarite, 248
Classroom demonstrations, 132
Cleaning fluid, 55
Cleansing procedures, 54
  contaminated materials, 57
  coverslips, glass, 55
  coverslips, mica, 57
  filters, various, 55, 56, 57
  flasks (Carrel), 55
  glassware, general, 55
  instruments, 57
  pipettes, serological, 57
  rubber goods, 57, 58
Clot digestion: and antitryptic agents, 173
  compensated for by patching, 156, 174, 220
  and horse serum, 169, 173
Cloth goods, 45
Coagulum, plasma: opacity of, 149
  washed to remove heterologous proteins, 170
Colchicine, 234

Cold storage, 7, 106
Collagenous fibers, 10
Colloid, 10
Conditioned medium (Earle), 26, 162
Connective tissue cells. *See* Fibroblasts.
Contaminated materials, disinfection of, 57, 66
Coverslip cultures, 132
  for assay purposes, 175, 176, 177, 219
  with double coverslips, 147
  and growth measurements, 219
  as hanging drops, 133
  in microcinematography, 268
  with perforated slides, 144
  with ring slides, 143
  for teaching, 132
  transplantation of, 138
Coverslips, mica, 46, 138, 239
  with stained cultures, 238
Cryoscopic (freezing-point) method of determining osmotic pressure, 86
Culture flasks: (Carrel), 43, 46, 148, 177, 179, 191, 219
  (Erlenmeyer), 204
  (Lindbergh), 191
Culture rooms: arrangement of, 40, 134, 217
  care of, 41, 70
Culture table, 41
Culture transplants, preparation of, 139, 159
Cystine, 117, 122, 125

Dakin's solution, preparation of, 71
Diack controls, 60
Diagnosis of tumors, 21
Dibenzanthracene, 18, 23
Differentiation, 10, 13, 215
Digestion of fibrin clots, 14, 220
  prevention of, 156, 169, 173
Diluting fluids, preparation of, 75
Double-coverslip cultures, 147
Dynamic medium (Gaillard), 13, 109

Ear, development of, 11
Earle's salt mixture, 80
Eggs (fowl) for culture work, 110
Electron miscroscope, 19, 172
Embedding procedures, 251
Embryology, experimental, 11, 215
Embryonin (Fischer), 121
Endometrium, 207
Epithelium, 10, 18, 118, 165, 166, 221, 226
Equipment, special, 43
Erlenmeyer flask cultures, 204
Extract, embryo tissue, 13, 107, 115, 119, 121, 122, 130, 149, 233
  and cell division, 108
  and differentiation, 13
  frozen and dried, 114
  and nucleoproteins, 107, 108, 233
  prepared by hand-chopping, 113
  prepared with Latapie apparatus, 114
  prepared with Pyrex homogenizer, 111
Eye, development of, 11

Face masks, 69
Feeding mixtures, 115
Fenwal flasks, 66
Fiberglas cloth, for protective pads, 64, 113
Fibrin digestion in clot: compensated for by patching, 156, 174, 220
  prevention of, 169, 173
Fibrinogen cultures, 88, 148, 170, 177, 220
Fibroblasts (connective tissue cells), 3, 4, 8, 10, 18, 118, 132, 159, 163, 174
  one year without subculturing, 149, 159
  races of, 9
  strains, development of, 163, 164, 165
  thirty-four-year-old strain, 3, 8, 107, 163, 226
Fibrosarcoma (myxoma), 22
Filters, various types, 67
  cleansing of, 55, 56, 57
  contaminated, 57
  sterilization of, 66
Filtration, 67, 81
Fixing reagents, 241
Flask techniques. *See also* Culture flasks.
  and adjustment of *p*H, 153, 194, 208, 212
  for assay purposes, 158, 163, 164, 165, 166, 175, 180, 187, 205
  for blood leucocytes, 166, 180
  for continuous circulation of fluid media, 190
  for fluid cultures, 33, 179, 204
  for long-continued cultures, 10
  with perforated cellophane, 26, 188, 195
  for plasma cultures, 148

Flask techniques—(*Continued*)
  and pure strains, 26, 160
  for tissues in suspension, 33, 204
Flasks: (Carrel). *See* Culture flasks
  (Erlenmeyer), 204
  (Fenwal), 66
  (Lindbergh), 191
Fluid cultures: for blood leucocytes, 180
  in Carrel flasks, 179
  with continuous circulation of medium, 190
  on coverslips, 144
  in Erlenmeyer flasks, 204
  in Lindbergh flasks, 190
  with perforated cellophane, 26, 188, 195
  for physiological studies, 205
  in roller tubes, 200
  for tissues in suspension, 204
  for virus propagation, 210
Formaldehyde, 241
Formalin, neutral, 241, 242
Freezing and drying, 88, 114
Freezing point (cryoscopic) determinations, 86
Fresh tissues, for assay purposes, 175, 210, 220
Fructose diphosphate, 124, 125, 128

Gas mixtures for flask cultures, 153, 194, 208
Gey's salt mixture, 81
Giemsa stain, 249
Glassware: cleansing of, 55
  contaminated, disinfection of, 57, 66
  sterilization, 64
Glucose utilization, 118, 228, 231
Glucosol solution, 79
Glutamine, 124, 125, 128, 129
Glycolysis, 124, 227, 230
Glycolytic cycle, 124
Gonads, 11
Green soap, tincture of, 70
Growth: energy, residual, 18
  factors, accessory, 116, 129
  of malignant cells, rate of, 18
  measurements, 218
  organized, 215

Hand sterilization, 70
Hanging-drop cultures, 133
Heart, bleeding from, 99, 100
Helly's fluid (Zenker-formol), 243
Hematoxylin: alum (Delafield), 249
  alum (Harris), 245
  ferrous-ferric (Earle), 246
  stain: for fluid cultures, 248
    for sections, 254
    for whole plasma cultures, 243
Heparin, 98, 100, 102
Heterologous plasma, use of, 169
Histological procedures, 236
History of tissue culture, 1
Hodgkin's disease, 22
Homogenizers, for preparation of tissue extracts, 111, 214
Homoiografting, 14
Hormones, 14, 117, 119, 210
Horse serum, 128, 169, 170, 173, 201, 202, 207

Inclusion bodies, 31
Incubators, 48
Individuality, and serum, 35
Instruments, 48, 93, 216
  cleansing of, 57
  sharpening of, 57
  sterilization of, 65
Insulin, 117, 119
Interference phenomena (viruses), 32
Iodine, tincture of, 252
Isotonic solutions, 86
Isotopes, radioactive, 235

Joint formation, 11
Jugular vein, bleeding from, 101

Laboratory, 39
  arrangements, 39, 134
  care of, 41
  Carrel's, 39
  organization, 54
  Pavlov's, 39
Lactic-acid production, 118, 224, 227, 230
Leucocytes, 2, 10, 118
  as biological test material, 166, 180, 187
  in fluid media, 180
  in plasma coagulum, 166
  normal and pathological, in various sera, 183
  staining procedures for, 256
Limb-buds, 11
Lipids, 91, 116

Liposarcoma, 22
Lymph, clotted, as used by Harrison, 3
Lymphosarcoma, 22
Lyophil process, 114

Macrophages. *See* Leucocytes
Malignant cells, 16, 21, 22, 115
Mammary gland tumors, 16, 149
Manometric (Warburg) determinations, 226
Masks, face, 69
Masson's trichrome stain, 245
Mayer's egg albumen mixture, 253
Measurements, growth, 218
Media: balanced salt solutions, 76
  conditioned (Earle), 26, 162
  continuous circulation of, 190
  dialyzed, 122, 123, 124, 125, 126
  diluting fluids, 75
  fluid, 26, 28, 144, 145, 146, 147, 179, 220
  gaseous atmosphere, 153, 191, 208
  heterologous, 169
  natural, 19, 75
  osmotic pressure, 86
  *p*H adjustments, 153, 191, 208
  *p*H determinations, 83
  plasma, 88
  serum, 88, 102
  synthetic, 18, 115
  tissue extracts, **13, 107**
Metabolism, measurements of, 226
Metabolites, intermediary, 116, 129
Metanephros, 11
Methylcholanthrene, 23, 25
Microcinematography, 268
Micrometer, ocular, measurements, 218
Microscope: electron, 19, 172
  inverted, advantages of, 260
Milieu intérieur (Claude Bernard), 1
Mitotic coefficients, 223
Mitotic index, 188
Modulations (Bloom), 10, 164
Monocytes. *See* Leucocytes
Myxoma, 22

Nerve cells, 22
Nerve fibers, development of, 2
Neural induction, 14, 26
Neurilemmoma, 22
Neuroblastoma, 22
Nitrogen metabolism, 118
Nucleic acid phosphorus, 232
Nucleoproteins, 107, 108, 121, 232
Nutrition, cell, 18, 115

Oil: of cedarwood, Florida extra, 240
  spindle, for dust prevention, 41
Organ rudiments, development of, 11, 215
Organized growth, 215
Osmotic pressure, freezing-point method for determining, 86
Ova, rabbit, development of, 13
Oxygen: consumption, 227
  for cultures with circulating media, 192
  for general flask cultures, 154
  for tissues in suspension, 213, 214

Papilloma (Shope), 19
Paraffin: for coverslip cultures, 137
  for embedding, 251
  for plasma tubes, 88
Parathyroid grafts, human, 14
Parlodion-methyl-benzoate solution, 253
Parthenogenesis, 14, 26
Patching, to compensate for digestion of coagulum, 156, 174, 220
Pepsin, to digest plasma coagulum, 225
Peptones, 116
Perforated cellophane cultures, 26, 188, 195
Perforated slides, for coverslip cultures, 144
*p*H:
  adjusted with exhaled air, 155
  adjusted with gas mixtures, 143, 153, 194, 201, 208, 212
  determined by drop method, 83
  determined by potentiometer, 83
  standards, 83, 85
Phase-contrast microscopy, 265
Phenol red: for cultures and standard colorimetric flasks, 85, 151, 153, 156, 170
  for determining *p*H by drop method, 84
Phenolor (Squibb), 66
Phosphate buffers (Sörensen), 83
Photomicrography, 259
Pigment formation, 10
Pipettes, calibration of, 50
Placental cord serum, 105, 170, 202, 207

Planimeter, 218
Plasma coagulum: opacity of, 149
  washed to remove heterologous proteins, 170
Plasma cultures:
  in Carrel flasks, 148
  for embryological studies, 215
  in roller tubes, 202
  on coverslips, 132
Plasma, preparation of, 88
Plasmagenes, 26
Pressure regulator for Lindbergh flasks, 194
Projectoscope: for photomicrography, 262
  for surface measurements, 218
Proliferation: and differentiation, 10
  of malignant cells, rate of, 18
Protein degradation products, 107, 117, 232
Proteins, serum, origin of, 35
Proteoses, 107, 116
Protocols. *See* Records
Pump flask (Carrel), 191
Pure strains from single cells, 26, 160

Racks, metal: for D-3.5 flasks, 51
    for pipettes and instruments, 43, 51, 133
  wooden: for coverslip cultures, 51, 146, 147
    for Erlenmeyer flasks, 208
    for glass tubes being filled, 44
    for glass tubes in storage, etc., 44
Radioactive isotopes, 235
Records, culture, 142
Regeneration, 1
Residual growth energy, 18
Rhabdomyosarcoma, 22
Ring-slide cultures, 143
Ringer-formol, 241
Ringer-Locke solution, 77
Roller-tube cultures, 196
  with plasma, 202
  without plasma, 200
Rotators, for roller tubes, 196
Rubber goods: cleansing of, 57, 58
  sterilization of, 65

Salt solutions, balanced, 76
Sarcoma, 2, 16, 18, 117, 162, 163, 174, 183, 226, 228
Schwann cells, 22
Sealing material. *See* Waterproof cements
Sealing mixture for coverslip cultures, 143, 145
Sectioning cultures, technique of, 250
Serum: individual variations in, 206
  preparation of, 88, 102
Serum cultures, one year without subculturing, 149, 159
Serum proteins, origin of, 35
Serum ultrafiltrate (Simms), 120, 212
Shipment of tissues, 102
Silver impregnation stain (Bodian), 255
Simms' salt mixture, 80, 212
Single-cell isolation, 26, 160
Skeletal rudiments, 11
Skin grafts, human, 1
Slide cultures. *See* Coverslip cultures
Soap: for cleansing glassware, etc., 55
  green, tincture of, 70
Solutions, sterilization of, 65, 67
Specificity: species, 29, 36
  virus, 29
Spindle cells. *See* Fibroblasts
Spindle oil, for dust prevention, 41
Staining procedures, 236
Sterilization: of brushes, nail, 65
  of cloth goods, 65
  detectors, 60
  by dry heat, 62
  of filters, various, 66
  of glassware, 64
  of hands, 70
  of instruments, 65
  by moist heat, 58
  of paraffin for plasma tubes, 88
  preparation of load, 61, 63
  principles of, 58
  of rubber goods, 65
  of solutions, 65, 67
Sternum, 11
Storage (cold) of tissues, 7, 106
Strains, pure, from single cells, 26, 160
Strepogenin (Woolley), 120
Structures, organ-forming, 11, 215
Sub-culturing, 138, 159
Suction: for filtration, 69
  for removing culture fluids, 156
Surface areas, measurement of, 218
Sympathicoblastoma, 22
Synthetic media, development of, 18, 164, 207

Teaching, 132
Technical assistance, 51
Test material, biological, 10, 109, 158, 163, 164, 165, 166, 175, 180, 183, 187, 210
Test tubes, as culture chambers, 196
Thrombin, 172
Thyroid grafts, human, 14
Thyroxine, 117, 119
Tissue homogenizers, 111, 214
Tissues: for shipment, 102
  for storage, 7, 106
Tooth germs, 11
Trace elements, 124, 127, 130
Transferring cultures: in flasks, 159
  on coverslips, 138
Transformation: of cell types, 10
  normal to malignant cells, 22, 162
Transplantation (grafting) of tissues, 1, 2, 14, 21, 26
Trimming cultures at transfer, 139, 159
Tube cultures, 196
Tubule formation, 10
Tumors: cultivation of, 2, 16, 149, 163, 166
  diagnosis of, 21
  milk-factor, 19, 20
  transplantable, 16, 21, 163
  virus-like agents in, 19
Tyrode's solution, 77
  without bicarbonate (glucosol), 79
  of double strength, 79
  with extra glucose, 79
  preparation of, 77
Viruses:
  chicken sarcoma (Rous), 19, 27, 30
  foot-and-mouth disease, 29, 30
  fowl plague, 29, 30
  fowl pox, 29
  GD-VII (Theiler), 212
  herpes, 31
  and immunity, 31
  inclusions, 31
  influenza, 32
  interference phenomena, 32
  lymphogranuloma, 31
  and milk-factor tumors, 19, 20
  propagation of, 27
  psittacosis, 31
  rabbit papilloma (Shope), 19
  rabies, 212
  specificity, 29
  vaccinia, 27, 28, 31, 32, 204
  Virus III, 31
  West Nile, 29, 32
  yellow fever, 28, 32, 212
Vitamins, 116, 118, 128, 129

Watch-glass cultures, 13, 215
Waterproof cements, 198
Weight (dry) of cultures, 224, 229, 231, 232
Wing, chicken, bleeding from, 97
Wrapping and packing for sterilization, 63

Zenker-formol (Helly's fluid), 243
Zenker's fluid, 242
Ziehl-Neelson's carbol-fuchsin, 258

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